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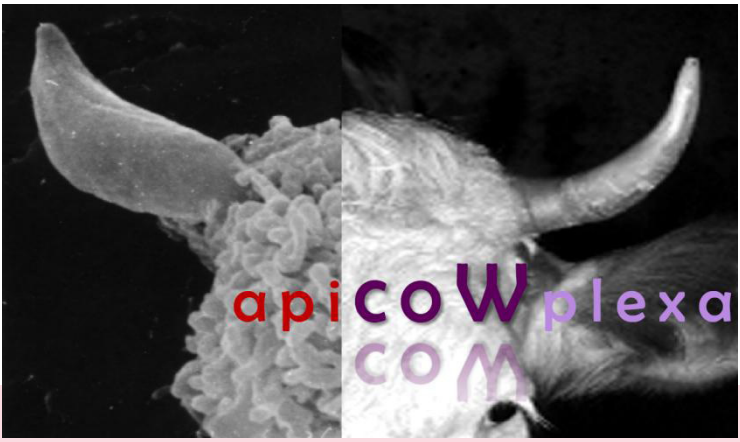

APICOMPLEXA IN FARM ANIMALS

International meeting · Lisbon, 25-28 October 2012

ApiCOWplexa
Apicomplexa in farm animals

PROCEEDINGS

Escola Superior de Tecnologia da Saúde de Lisboa

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Welcome

Apicowplexa 2012: International Meeting on Apicomplexan Parasites in Farm Animals

October 25-28, 2012
Lisbon

Dear colleagues,

A warm welcome to Apicowplexa 2012, an international scientific meeting that is dedicated to apicomplexan parasites in farm animals!
Apicomplexans cause a variety of diseases in animals and also in humans. However, while human-pathogenic diseases caused by apicomplexans are relatively well covered in terms of meetings and scientific exchange, those apicomplexans causing diseases in farm animals have been largely left out. Thus, this is why this meeting has been initiated: we want to bring together academics, researchers, students and industrial partners working on apicomplexan parasites in farm animals, and provide this meeting as a platform for scientific exchange, which is instrumental for successful networking and meeting potential collaborators. In addition, we want to discuss the possibilities and steps that could be taken in order to improve scientific exchange and collaborations, and we hope that you will participate and share your views with your colleagues.
We thank those who have accepted our invitation to support this meeting by attending and providing posters, oral presentations or keynote lectures.

In any case, we wish you an excellent meeting and a very enjoyable time in Lisbon!

The Organizing Committee

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Programme

Thursday 25th October, 2012

15:00 **Registration and posters display**

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Opening lectures

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18:00 Keynote — David S. Roos (University of Pennsylvania, USA) "Mining the 'omics data deluge to expedite discovery research"

18:30 **Welcome reception**

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09:00 — Daniel Gutiérrez Expósito "Herd and individual seroprevalence of *Besnoitia besnoiti* infection and associated risk factors in beef breeding cattle in an endemic region of the Spanish Pyrenees"

09:15 — Philippe Jacquiet "Is it possible to stop the spread of bovine besnoitiosis in areas of emergence?"

09:30 — Nicole Gollnick "Transmission of *Besnoitia besnoiti*: Close contact of cattle plays key role"

09:45 — Khuanchai Koompapong "Identifying the sources of environmental contamination by *Cryptosporidium* spp."

10:00 Renato Andreotti “Economic impact of neosporosis on productive system of beef cattle in Mato Grosso do Sul State, Brazil”

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10:30 Fernando Paiva “*Eimeria* species (Apicomplexa: *Eimeriidae*) in beef cattle and sheep in Mato Grosso do Sul State, Brazil”

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12:15 Furio Spano “Integrating global proteomics and single protein analysis towards the understanding of the biology of the *Toxoplasma gondii* oocyst/sporozoite”

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Chair: Brian Cooke & David Allred

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Chair: Bruno Gottstein & Gereon Schares

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16:30 Keynote	Luis Ortega-Mora (Universidad Complutense de Madrid, Spain) "Vaccine development against bovine neosporosis: present situation and *in vitro* and *in vivo* models to test safety and efficacy"

17:00	John Ellis “Efficacy and safety of vaccination in cattle with live tachyzoites of *Neospora caninum* for the prevention of Neospora-associated fetal loss”
17:15	Silvia Rojo-Montejo “Effect of vaccination of cattle with the naturally attenuated Nc-Spain 1H isolate of *Neospora caninum* on responses to heterologous challenge during early and mid gestation”
17:30	Pascal Breton “VitamFero: Novel proprietary live attenuated vaccines against Apicomplexa”
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18:00	Giles Gargala “Chlorothiazolides as effective anticryptosporidial agents *in vitro* and in immunosuppressed gerbils”
18:15	Gisela Greif “Target identification of toltrazuril – review of recent results”
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Sunday 28th October, 2012

9:00	**Round table discussion: summary, feedback and conclusions** **Chair: Alexandre Leitão & Andrew Hemphill**
10:00	**Closing session**
10:30	Coffee

Opening lectures

Antigenic diversity in *Theileria parva* and the basis of escape from immune recognition

W. Ivan Morrison, Tim Connelley, Johanneke D. Hemmink, Xiaoying Li, Niall D. MacHugh

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Theileria parva is a parasite of African buffalo (*Syncerus caffer*) and cattle, found throughout a large part of East and Southern Africa. It is highly pathogenic in cattle, but animals that recover from infection are solidly immune to challenge with the same parasite strain. However, *T. parva* is one of several protozoan parasites that display strain-restricted immunity. Experiments involving cell transfer between twin calves have demonstrated that CD8 T cells are important mediators of immunity and the strain specificity of CD8 T cell responses in immunised animals has been shown to correlate with immune status following heterologous parasite challenge. The recent identification of a series of parasite proteins recognised by immune CD8 T cells has enabled us to investigate both the antigenic diversity in parasite populations and the basis of strain-restricted immunity. Our results indicate that immunodominance of the CD8 T cell response in individual animals, where the majority of the response is focused on one or two dominant antigens, is a key factor in determining strain specificity. The particular antigens that dominate the response depends on the host class I MHC type. Analyses of sequences of genes encoding the CD8 target antigens have revealed that some antigens are highly polymorphic whereas others are almost completely conserved. Available data indicate that the former tend to generate dominant responses. These findings, coupled with evidence that populations of *T. parva* in the field frequently undergo sexual recombination, indicate that merely broadening the antigenic specificities of CD8 T cell responses would be sufficient to overcome the problem of strain restricted immunity. Comparison of parasites maintained in cattle in the absence of buffalo with those derived from buffalo have revealed much more extensive diversity in buffalo-derived parasites, indicating that the cattle-maintained parasites represent a subpopulation that has become adapted for transmission between cattle.

Mining the 'Omics data deluge to expedite discovery research

David S. Roos* ... on behalf of the EuPathDB team

University of Pennsylvania, Philadelphia PA 19104, USA.

Biomedical research is increasingly driven by large-scale datasets: genome sequences, RNA and protein expression results (generated on diverse experimental platforms), population-level data on genetic polymorphisms and epidemiology, information on protein structure, interactions and subcellular localization, metabolic pathways and signaling networks, phenotypic descriptions of laboratory mutants/treatments and field/clinical isolates, *etc.* Infectious disease studies are further complicated by the interplay between pathogen, host, and vector species. ***Help!!!*** How can we effectively collect, store, maintain, integrate, and mine this information, so as to advance biological understanding, and define targets for further investigation in the lab, field and clinic?

The Eukaryotic Pathogen Genome Database (EuPathDB.org) provides researchers working on many apicomplexans of veterinary importance with convenient access to genomic-scale datasets, in a phylogenetic framework that expedites discovery research. In addition to offering both gene- and genome-centric views, and a mechanism for capturing expert annotation of genes and isolates contributed by the scientific community, a graphical user interface simplifies the formulation and optimization of complex queries. For example, investigators seeking to identify factors likely to modulate host responses to infection might wish to search for genes that are conserved in pathogenic but not non-pathogenic species, expressed in relevant strains and during appropriate life cycle stage(s), secreted by the parasite, harbor domains suggestive of interaction with host factors, and displaying signatures of evolutionary selection. Similar strategies might be employed to identify diagnostic markers of infection, therapeutic targets, *etc.* Such queries can be shared with colleagues or stored for future use, refinement, or modification, enabling systems-level analysis of biologically and clinically relevant problems. Various queries will be illustrated in this presentation, demonstrating how such *in silico* experiments can help to prioritize further research effort.

We will also discuss informatics challenges for the future, including the continuing onslaught of data and growing diversity of data types: microscopic and whole animal images, population diversity data, metabolomics, small molecule screening results, host- and vector-responses (including clinical information), etc., metadata standards for pathogens, patients, populations, and experimental and clinical studies (ideally using structured ontologies that capture meaningful information); and new tools for capturing, analyzing, integrating, and mining emerging datasets.

Epidemiology and economic impact

Does *Neospora caninum* have economic impact? – the billion dollar question

Michael P. Reichel[1,2] and John T. Ellis[2,3]

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2 School of Med. & Molecular Biosciences, University of Technology, Sydney, PO Box 123, Broadway, NSW 2007, Australia;
3 i3 Institute, University of Technology Sydney, Broadway, Australia.

Neospora caninum is invariably rated as one of the most important infectious causes of abortions in cattle world-wide. In Australasia, where bovine brucellosis is absent now, it is usually reported to be the cause of 30-40% of all diagnosed abortions, particularly in dairy cattle. Despite that, there seems to be evidence now of a waning interest in the disease, possibly because the economic impact of the infection has not been clearly communicated to primary producers and members of the veterinary community.

A search was conducted on PubMed using *cattle* and *Neospora* as search terms. As of January, 31st, 2012, this yielded 769 publications whose abstracts were screened individually for suggestions of economic relevant information (abortion incidence, prevalence and risk, serological data, impact on milk production and reproductive parameters). Countries with at least five relevant publications were included and subjected to further analysis. In total, 99 studies (12.9%) from ten countries, containing data that pertained to a total of 221,713 head of cattle, of which 45,863 (20.7%) resided in the beef industry were included in the final analysis. 25 papers (25.3%) contributed data from the beef industry and 72 papers (72.8%) from the dairy industry (with the remaining two papers (2.0%) describing general abortion statistics).

The analysis of this available data from the publications that contained economically relevant data showed the most prominent economic impact of *N caninum* infections to be that of abortion events. The total annual cost was estimated to range from a median US $1.119 million in the New Zealand beef industry to an estimated median total of US$ 546.3 million impact *per annum* in the US dairy population. The total annual costs in just those countries alone, was exceeding US$ 1 billion *per annum*.

A review of *Neospora* and cattle-related literature yielded limited published information on the economic impact of infection. Most of the qualifying articles dealt with individual case or herd reports, with limited controls or were studies that established sero-prevalence data only. Nevertheless, a structured analysis of the published literature provides a first global economic impact of neosporosis arriving at an estimate in excess of one billion US dollars annually from just nine countries alone. This estimate, with the identification of nine clear target markets, should provide renewed incentive to the animal pharmaceutical companies to develop treatment options and/or vaccines.

Herd and individual seroprevalence of *Besnoitia besnoiti* infection and associated risk-factors in beef breeding cattle in an endemic region of the Spanish Pyrenees

Gutiérrez-Expósito D.[1], Estéban-Gil A.[2], Ortega-Mora L.M.[1], Castillo J.A.[2], García-Lunar P.[1], Álvarez-García G.[1]

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A recent expansion of bovine besnoitiosis has been reported based on an increase of clinical cases recorded in different European countries in the last few years. Accordingly EFSA authority has pointed out the necessity of conducting prevalence studies to determine the expansion and importance of the disease. In the present study, herd, within-herd and individual prevalences for *Besnoitia besnoiti* infection together with risk factors associated to it were determined in beef cattle located in a traditional endemic region from Huesca, one of the provinces located in the Spanish Pyrenees. A total of 3798 animals older than 1 year were collected. Sera from females (n=3211; error margin of 1.08 % according to female beef cattle census) and males (n=587; total census sampled) were sampled independently. Females belonged to 63 herds where at least 50% of animals were sampled (error margin of 11.5% according to beef herd census). Then intra-herd, herd seroprevalence as well as individual female prevalence were estimated. On the other hand, all males belonged to 307 herds and individual male prevalence was calculated. All herds practice natural mating and share grazing pastures in summer in high altitudes where bloodsucking arthropods and wild ruminants are present. Individual risk factors studied were age and sex. Sera were tested by Enzyme-Linked Immunosorbent Assay (ELISA) (97% Se and 95% Sp) and a herd was considered seropositive if at least one animal was seropositive. Single or two reacting animals in a herd by ELISA were confirmed *a posteriori* by western blot. Herd prevalence rate was 87.30 % and intra-herd prevalence rates varied greatly between 15.1 and 95.7%. Both sexes were similarly affected (48.74%, 95%CI: 45-52%) of seropositivity in males *versus* 51.86% (95%CI: 50-53% in females) ($P>0.05$, χ^2) and a significant increase of seropositivity with age was observed (10.27%, 95%CI: 7-12%) in 1-3 years-old animals raised up to 76.65% (95%CI: 74-78%) in > 7 years-old animals ($P<0.001$, χ^2). To our knowledge, this is the first prevalence study carried out in males. Moreover the results in both sexes evidence that bovine besnoitiosis is highly widespread in beef cattle from Pyrenees. Thus, serological examination is highly recommended when there is beef cattle trade from endemic to *Besnoitia*-free regions.

Is it possible to stop the spread of bovine besnoitiosis in areas of emergence?

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Background: *Besnoitia besnoiti* is the causative agent of bovine besnoitiosis. Mechanical transmission by horse flies and stable flies is the main route of contamination. Historically present in the French Pyrenees, bovine besnoitiosis shows a recent spread in the Alps and in the Massif Central (South of France). In these areas of emergence, only a relatively small part of the cattle flocks are infected. As no effective treatment and no vaccine are yet available in France, the unique option to control bovine besnoitiosis is the selective culling of seropositive animals.

Protocol: In order to confirm the interest of this option, a recently infected area was chosen in the south-east of Massif Central comprising eleven adjoining beef-cattle farms. Neither common pastures nor commercial exchanges of cattle between these farms occur. Clinical cases of bovine besnoitiosis were observed for the first time in 2006 in one farm and in five other farms in 2009. In March 2010, serological prevalences were evaluated by ELISA (PrioCheck Besnoitia Ab. 2.0) and Western Blot. In four farms, the prevalences were low (from 1.2 to 5%) and exhaustive culling of seropositive animals was applied. Higher prevalences (from 13.6 to 57.6%) were noted in the seven remaining farms where only regular applications of insecticides were proposed. Annual incidences of *B. besnoiti* infections were evaluated by serology in March 2011 and March 2012.

Results and conclusions: No seropositive animals were detected in 2011 and 2012 in the four farms where an exhaustive culling was previously done. Serological incidences were high (from 10 to 80%) in the adjoining seven farms during the same period. The rapid elimination of seropositive animals in lowly infected farms seems to be an efficient option to stop the spread of bovine besnoitiosis even if active "intra" herd transmission occurs in neighboring farms. Here, the absence of "between" farms transmission is likely due to the feeding behavior of vectors and to the mechanical nature of transmission.

Transmission of *Besnoitia besnoiti*: Close contact of cattle plays key role

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A 12-week cohabitation study was conducted in order to investigate the effect of spatial separation of *Besnoitia besnoiti* affected and healthy cattle. In addition, the influence of mating was evaluated. Furthermore, insect activity with regards to mechanical transmission of the parasite was studied by collecting insects alighting on or flying in the vicinity of cattle and examining these arthropods for *B. besnoiti* DNA. Five German Simmental heifers and a German Simmental bull were kept on pasture together with three non-pregnant Limousin cows with chronic bovine besnoitiosis. Two Limousin cows in the acute stage of disease were introduced into this pasture group on days 3 and 51 of the experiment. Throughout the study, a group of six German Simmental heifers were confined in a paddock at a minimal distance of 20 meters from the pastured animals. Clinical exams were performed daily on the 12 Simmentals and blood and tissue samples were collected twice a week for PCR, histological and serological investigations. Sampling frequency was increased in animals which became infected during the investigation period. Naïve cattle kept in the paddock did not become infected with the parasite. However, three heifers in the pasture group developed antibodies against *B. besnoiti* during the observation period. Two of these animals displayed clinical signs of acute bovine besnoitiosis around the time of seroconversion. Four insect species were caught in this study, *Musca domestica*, *Musca autumnalis*, *Haematobia irritans,* and *Stomoxys calcitrans*, and *B. besnoiti* DNA was detected in one of 48 *S. calcitrans* flies caught directly on an acutely infected heifer. Infection of heifers was correlated with frequent sampling of *B. besnoiti* infected cattle in early stages of disease, which already harbored parasitic cysts in their dermis. Intensive mating activity, however, neither led to infection of the bull nor correlated with infection of female cattle.

Identifying the sources of environmental contamination by C*ryptosporidium* spp.

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Cryptosporidium oocysts contaminating water are potential cause of important waterborne outbreaks that impact world health and economies. Knowing the sources of contamination allows for management methods. In the case of *Cryptosporidium*, besides knowing the source location, one must also determine the oocyst identity to ascertain if the humans or animals are the sources responsible. The study's aims were to explore prevalence of oocyst contamination along with seasonal variation and host factors in Chao Phraya River and seawater of Bang Pu, Samut Prakan Province, Thailand. Altogether, in 2010-2011, 144 water samples were collected from Chao Phraya River (72) during summer, rainy, and cold season, and 72 samples from seawater before, after, and during the presence of migratory seagulls. Due to birds being potential sources, 70 fecal samples from Bangkok's pigeons and 910 from migratory seagulls at Bang Pu Nature Reserve pier were also collected. There were 11.1% and 5.6% of samples positive by nested-PCR from river and seawater, respectively. The highest river contamination was in the cold season, and seawater contamination was highest when seagulls were present. Other than that, 14.3% Bangkok's pigeons and 15.4% seagull fecals samples were positive. The sequencing results revealed all oocysts from Chao Phraya River were *C. parvum*. For seawater, 50% of identified oocysts were *C. parvum*, 25% *C. serpentis*, and 25% *C. meleagridis*. The one positive sample of Bangkok's pigeons was *C. meleagridis* and all of positive seagulls were *Cryptosporidium* avian genotype III. Oocyst from both water and birds samples were 55.6% viable using a dye permeability assay. Humans, farm animals, and wildlife were suspected as the sources of *C. parvum* and *C. serpentis* in the water of this study. Rainwater runoff likely carries the oocysts of *C. meleagridis* from Bamgkok's pigeons and other birds into the ocean. Migratory seagulls are potentially importing *Cryptosporidium* avian genotype III into Thailand from China. This is the first molecular study of *Cryptosporidium* in Thai waters and the first report of *C. serpentis* and *Cryptosporidium* avian genotype III in Thailand.

Economic impact of neosporosis on productive system of beef cattle in Mato Grosso do Sul State, Brazil

Renato Andreotti*, Jacqueline Cavalcante Barros

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This work aimed to evaluate the economic impact of the neosporosis occurrence on productive sector of beef cattle that plays a significant role in developing the economy of the Mato Grosso do Sul (MS) State, Brazil. The search for new world markets has led Brazil to worry about quality standards, lower environmental impact, tracking, competitive price, and the sanitary requirements. The study of a disease under an economic standpoint is justified to estimate the loss that the disease brings to rural activities, and scale it to the state of MS. Were evaluated 1098 heifers from the breeding season to the birth of calves in relation to reproductive performance, and it was performed the serological diagnosis of neosporosis. Using the Gerenpec software was simulated the evolution of their livestock and farm income for a second modal pattern technology as well as for the State. The birth rate for heifers seropositive and seronegative for neosporosis was 28.24% and 50.12% respectively. The results identified a significant association between heifers positive for neosporosis and the proportion of abortions in the herd, which caused an impact on reproductive performance of 6%. Properties that embrace low-tech practices suffered an economic loss with the disease of 14%, in the properties with the use of average technology the loss was 21% and those who adopt high-tech the loss was 34%. The neosporosis caused a negative impact on the collection of ICMS (state tax), by economic activity of cattle, 25% for the period of 10 years, which corresponds to values of 2009, a loss in revenue of R$ 46,046,037.06. The study of the disease, under the economic point of view, can develop strategic actions in the management of the property considering the financial return, as well as control of disease in the State.

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Serosurveillance of *Toxoplasma gondii* infection in sheep, bovine and goats of France

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Toxoplasma gondii is generally transmitted by ingesting tissue cysts from undercooked or raw meat or consuming food or drink water contaminated with oocysts. A nationwide study was conducted to evaluate the prevalence of *T.gondii* in fresh ovine and bovine meat, while a serological survey of the goat population of the departments of Aube and Marne was performed.
Diaphragms and hearts from 433 sheep and 2349 bovine were collected and analyzed in correlation with their age, sex and geographical origin. A total of respectively 398 and 570 muscles samples (diaphragms) originating from imported sheep and bovine carcasses were also included. Fluids from hearts and diaphragms were tested serologically. Direct detection of parasites was performed by mouse bio-assay. Moreover, 412 goat serums were collected from 23 farms (1-51 goats/farm) originating from Aube and Marne (East of France).
The overall estimate of *Toxoplasma* seroprevalence was 17.7% (11.6-31.5%) for lambs, 3% (2-5%) for calves and 89% (73.5-100%) for adult sheep, respectively 18% (16-20%) for adult bovines ($P<0.0001$). No significant difference was observed between imported and French meat. In France, seroprevalence in lambs showed an increasing North-western to Southern gradient. The proportion of French carcasses carrying live parasites according to bioassay results was of 46/433 (45 genotype II; one genotype III) for sheep carcasses and 2/2349 (2 genotype II) for bovine carcasses. For the goat populations of Aube and Marne the mean prevalence was 34,2% (141/412), ranging from 0% (0/51) to 100% (4/4).
This study offers an accurate drawing of the toxoplasmosis pattern amongst sheep and bovine consumed in France, and a model for a zoonosis hazard. control survey.

Eimeria species (Apicomplexa: *Eimeriidae*) in beef cattle and sheep in Mato Grosso do Sul state, Brazil

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The coccidium *Eimeria* spp. infects many vertebrate species, mainly farm animals. Young animals are more susceptible to the clinical disease while older animals act as carriers, disseminating the agent into the environment. The species can be differentiated by morphology and morphometry of oocysts, sporocysts, sporozoits and other special characters, such as cell wall, polar cap, polar granules and residual body. In Brazil there are few studies on the occurrence and prevalence of eimeriosis. The species involved in ruminant infection in the diverse regions of the country have yet to be fully characterized. This study aimed to identify the *Eimeria* species in naturally acquired infections in cattles and ovines in the State of Mato Grosso do Sul, Brazil. We collected feces samples from animals in the counties of Camapuã, Campo Grande, Corumbá, Coxim, Ivinhema, Jaraguari, Naviraí, São Gabriel do Oeste, Sidrolândia, Terenos and Três Lagoas. The occysts were quantified by determining the Oocyst Count per Gram (OoPG) by utilizing the modified Mc Master technique. The modified Sugar Flotation (Sheather's technique) was used to identify the *Eimeria* species. The species found in bovines included: *E. alabamensis* (5%), *E. auburnensis* (12%), *E. bovis* (37%), *E. brasiliensis* (6%), *E. bukidnonensis* (1%), *E. canadensis* (27%), *E. cylindrica* (3%), *E. ellipsoidalis* (4%), *E. subspherica* (1%) and *E. zuernii* (4%). For ovines: *E. ahsata* (3%), *E. arloingi* (17%), *E. bakuensis* (6%), *E. crandallis* (21%), *E. ovinoidalis* (8%), *E. pallida* (15%), *E. parva* (19%) and *E. weybridgensis* (11%). All of these species have a global distribution. Morphologic characteristics of oocysts and sporocysts allow an adequate and practical discrimination of *Eimeria* species. With the information collected from the aforementioned properties and the results obtained in this study we observed that *Eimeria* spp. is widespread in the state and the subclinical form of the disease predominates.

Functional genomics and gene expression

Close relatives reveal family secrets in the Apicomplexa - a systems approach to understanding the genomes of *Neospora* and *Toxoplasma*

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Neospora caninum and *Toxoplasma gondii* are closely related tissue-dwelling Coccidian parasites that share many common morphological and biological features. Despite many similarities, they differ dramatically in their host-range and definitive hosts: exclusively felids in *Toxoplasma*, but exclusively canids in *Neospora*. Unlike *Toxoplasma*, *Neospora* appears not to be zoonotic, having a more restricted host range in which it occupies a unique ecological niche showing a striking capacity for highly efficient vertical transmission in bovines. *N. caninum* is one of the leading causes of infectious bovine abortion, resulting in significant economic losses to the dairy and beef industries. We have undertaken a comparative analysis of the genomes and transcriptomes of these two species and also investigated comparative host-parasite interactions using a systems biology approach. We propose that speciation of *Neospora* and *Toxoplasma* occurred some 28 million years ago, after that of cats and dogs. We have identified unusual gene family expansions, pseudogenisation events in key host invasion-related genes and changes in gene regulation. These suggest that evolution has focused on molecules that control the interaction of the parasite with the host cell. Specifically we demonstrate that key rhoptry genes and surface genes which control virulence and host cell interactions in *Toxoplasma* are dramatically altered in their expression and functionality in *Neospora* and propose that evolution of these genes determines the ecological niches inhabited by these parasites.

The *Theileria* schizont: clever scavenger and host cell modulator

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The protozoan parasite *Theileria* possesses the unique capacity to immortalise the leukocytes it infects. This results in a lymphoproliferative disease that is characterised by the clonal expansion of the parasitized cells. The transforming schizont stage resides free in the host cell cytoplasm where it manipulates host cell signal transduction pathways that regulate proliferation and cell survival. The schizont is strictly intracellular and to maintain transformation, the parasite must be distributed over the two daughter cells each time the host cell divides. To guarantee the faithful distribution over the two daughter cells, the schizont first interacts with host cell astral microtubules and, as the cell exits mitosis, becomes incorporated in the central spindle, in a process that involves the recruitment of host cell Plk1 to the parasite surface. We observed that newly formed host cell microtubules become stabilised at the parasite surface, but the proteins involved in this process have not yet been described. Microtubules are highly dynamic structures subject to elaborate spatiotemporal regulation and the mechanism by which the parasite interacts with host cell microtubules is still largely unknown. Mass spectrometric analysis of purified schizonts resulted in the identification of two proteins, previously reported to be expressed by sporozoites. Using a conserved binding motif, one of these proteins, p104, was found to interact in a cell cycle-dependent manner with a host cell protein that has a central role in regulating microtubule dynamic instability. A second host cell protein that regulates microtubule stability also locates to the parasite surface. Our findings provide first insight into the molecular basis for schizont persistence in the continuously dividing host cell.

Integrating global proteomics and single protein analysis towards the understanding of the biology of the *Toxoplasma gondii* oocyst/sporozoite

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Given the central epidemiological role played by the *Toxoplasma gondii* oocyst/sporozoite and the limited knowledge on its biology, we have recently carried out a global proteomic survey of this parasite stage using one-dimensional gel electrophoresis coupled to liquid chromatography-linked tandem mass spectrometry. The analysis of total or fractionated protein extracts obtained from partially sporulated oocysts of the *T. gondii* strain VEG (genotype III) yielded a dataset of 1685 non reduntant proteins, accounting for ~21% of the total predicted proteome. Approximately 35% of the identifications corresponded to hypothetical proteins, whereas 54% were functionally classified. Importantly, the comparison of the VEG oocyst dataset with the extensively covered proteome of the *T. gondii* tachyzoite identified 211 putative oocyst/sporozoite-specific proteins (POSP), some of which were validated by Western blot analysis. The functional profile of the POSP subset is consistent with the adaptation of *T. gondii* oocysts to the nutrient-poor and stressing extracellular environment, as shown by the preponderance of enzymes involved in metabolism and energy production or by the presence of a photolyase DNA repair enzyme. Proteomic data showed that, compared to tachyzoites, oocysts have a greater capability of de novo amino acid biosynthesis and are well equipped to fuel the Krebs cycle with the acetyl-CoA generated through the β-oxidation of fatty acids and the degradation of branched amino acids. In addition, our data indicated that *T. gondii* sporozoites and tachyzoites possess extensively overlapping repertoires of invasion-related proteins, yet sporozoites express a broader complement of proteins involved in the formation of the moving junction, including paralogs of the mutually interacting AMA1 and RON2 molecules. Additionally, we have undertaken the characterization of select secretory proteins expressed preferentially or exclusively in the oocyst/sporozoite of *T. gondii*, including oocyst wall components, a family of LCCL domain-containing molecules and a mucin-like protein encoded by a multigene locus.

De novo genome assembly from DNA sequence capture of *Theileria parva*, an apicomplexan parasite of cattle in sub-Saharan Africa

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East Coast fever, which occurs in eastern, southern and central Africa, is an acute fatal disease of cattle caused by the tick-transmitted intracellular apicomplexan pathogen *Theileria parva*. The lack of a stable, inexpensive and logistically straightforward immunization method to protect against this parasite makes the development of an effective recombinant vaccine a high priority. In the last few years reverse vaccinology, based on whole genome sequence and population genomics data, has emerged as a primary approach to identify putative vaccine antigens from genome sequence data. The application of reverse vaccinology to *T. parva* presents several fundamental challenges, starting with the isolation of parasite DNA for genome sequencing without sacrificing the bovine host. Here we report the successful capture and sequence of *T. parva* genomic DNA from a *T. parva*-infected lymphocyte cell line. The capture probe set was designed to cover 95% of the 8.3 Mb genome of the *T. parva* Muguga strain, including 98% of its annotated genes. We built both 454 and Illumina libraries from total DNA isolated from bovine lymphocytes infected with the Muguga isolate. Sequence reads from DNA fragments captured from both types of libraries mapped to >95% of the *T. parva* Muguga genome. Unmapped reads totaled less than 25% of the captured reads, establishing the selective capture of *T. parva* DNA over that of the host. A genome assembly generated *de novo* from the sequence reads recovers 99% of the reference Muguga genome, and >99.5% of its genes. Preliminary results suggest that this approach can be used to capture genomic DNA from divergent *T. parva* isolates. This study demonstrate the feasibility of whole-genome sequence capture to generate population genomics data needed for reverse vaccinology of *T. parva*, and is directly applicable to a variety of intracellular pathogens, including other apicomplexans.

Microarray analysis of *Theileria annulata* infected cells reveals irreversible modulation of activation and neoplasia associated host cell gene expression profiles

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Infection of bovine leukocytes by *Theileria annulata* results in establishment of immortalised, infected cells. This event is known to involve constitutive activation of Janus-like pro-inflammatory transcription factors that have the potential to be beneficial or detrimental. Using microarray methodology we have generated gene expression profiles representing an immortalised uninfected bovine cell line (BL20 cells) and the *Theileria*-infected counterpart (TBL20). Comparative expression profiling was performed on these cell lines following treatment with the inflammatory mediator, LPS or treatment with BW720c to kill the parasite. While stimulation with LPS induced cell death and activation of NF-κB in BL20 cells, the viability of *Theileria*-infected TBL20 cells was unaffected. Analysis of expression networks showed that the parasite establishes tight control over pathways associated with cellular activation. This includes modulated expression of the TLR4 receptor for perception of LPS signalling and modified expression of the target genes of infection-activated transcription factors, including NF-κB. Treating the parasite infected TBL20 with BW720c failed to revert these cells to the immortalised uninfected BL20 phenotype and cell death was induced. This was mirrored by a failure to reverse infection-associated changes to gene expression at multiple levels. IPA analysis highlighted genes encoding transcription factors and modifiers of chromatin architecture as potential primary targets of the viable parasite. Our results provide evidence that *T. annulata* irreversibly reconfigures host cell gene expression networks associated with inflammatory disease and cancer to generate an outcome that promotes survival and propagation of the infected leukocyte.

First 2-DE approach towards characterising the proteome and immunome of *Besnoitia besnoiti* in the tachyzoite stage

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Bovine besnoitiosis is caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti.* It is considered to be a re-emergent disease in Europe and is also present in Africa and Asia. Due to the chronic and debilitating course of the disease, bovine besnoitiosis is responsible for severe economic losses. However, many aspects of the disease and parasite biology remain unknown. Proteomics studies could help to investigate relevant biological processes as well as host immune response associated with parasite infection. Both the proteome and immunome of the tachyzoite stage of *B. besnoiti* of the Bb-Spain1 isolate are described herein for the first time. Tachyzoite protein extracts were first separated by 2-DE SDS-PAGE using pH 3-10 NL IPG strips for Coomassie Brilliant Blue-stained gels and immunoblots. Eighty-five out of 265 spots visualised on Coomassie-stained gels were immunogenic when pooled serum from naturally infected cattle was used, and the distribution of immunogenic spots correlated with the 1-DE IDA pattern. Because most spots were found in the acidic range of the pH gradient, pH 3-6 L IPG strips were used next, and 58 out of 123 visualised spots proved to be immunogenic. Twenty-seven spots were identified by MALDI TOF/TOF to be 20 different proteins due to the presence of protein species. All proteins identified corresponded to highly conserved proteins among eukaryotes. Six proteins identified are related to energy metabolism, 3 are heat shock proteins, 4 proteins are related to host cell invasion processes, and 2 proteins are involved in cell redox homeostasis. A tryptophanyl tRNA synthetase, a putative gbp1p, nucleoredoxin, a putative receptor for activated C kinase, and a nuclear movement domain-containing protein were also identified. Among these proteins, fructose-1,6-bisphosphate aldolase, lactate dehydrogenase, pyruvate kinase, enolase, HSP60, HSP70, HSP90, actin and profilin proved to be immunogenic, and 5 were cross-reactive antigens between *B. besnoiti* and *N. caninum*. This first proteomic approach carried out in *B. besnoiti* should be followed by other studies to identify more specific parasite proteins.

Recent advances in *Babesia* research

Molecular underpinnings of long-term persistence by *Babesia bovis*

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Despite their small genomes and a robust host immune response toward them, babesial parasites are capable of establishing persistent infections of extremely long duration. With a genome of less than 9 Mbp the bovine parasite, *Babesia bovis*, is an excellent example of this phenomenon. At least two mechanisms of persistence are employed by this parasite- cytoadhesion to the vascular endothelium and antigenic variation- allowing avoidance of splenic clearance and host antibodies, respectively. Central to both mechanisms is the variable erythrocyte surface antigen 1 (VESA1) encoded by the *ves* multigene family, perhaps with assistance from the similarly variable *smorf* multigene family. The *ves* family alone is comprised of approximately 150 members scattered in clusters about the genome, mainly in divergently-oriented gene pairs. This unusual organization raises questions regarding mechanisms underlying the monoparalogous transcription of this gene family. Moreover, this organization may play a role in a unique nucleosomal remodeling of *ves* genes for transcription, and the sequential appearance and assembly of VESA1 subunits on the infected-erythrocyte surface. In contrast, the 44 member *smorf* family is interspersed among the *ves* clusters as individual genes, but it remains unclear whether they are coordinately regulated with nearby *ves* genes. Antigenic variation in *B. bovis* appears to occur primarily through segmental gene conversion of a single actively transcribed *ves* locus; the organization of this family may influence the use of individual *ves* genes as sequence donors during this process. Recent efforts at understanding the transcriptional regulation of the *ves* multigene family and the machinery responsible for its sequence variation will be presented.

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Successful vaccination against *Babesia* with recombinant antigens

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Plasma from *Babesia*-infected animals has been shown to induce protection in naive animals when used as vaccine, suggesting that *Babesia* parasites release soluble antigens in the plasma during patent infection. After the development of *in vitro* cultivation of *Babesia* parasites it could be shown that supernatants of such cultures also induced protective immunity in naïve hosts, corroborating the hypothesis that *Babesia* parasites release soluble parasite antigens in the environment. Detailed analysis of the supernatant of *B. divergens* cultures revealed that a Mr 37 kDa parasite molecule was the main immunogenic component (Bd37). The gene was cloned and expressed in *E. coli* as a recombinant GST fusion protein. Vaccination-challenge studies in gerbils showed that the antigen induced complete protection against virulent *B. divergens* challenge infection. Protection was reflected in decreased parasitaemia. It was further shown that Bd37 is GPI-anchored at the merozoite surface. Using *B. canis* in the dog it was shown that vaccination of dogs with supernatants of *B. canis* cultures induced immunity against homologous challenge infection. Importantly, serum from dogs that were vaccinated and subsequently challenged showed a unique specificity that was different from that of non-vaccinated dogs that survived a *B. canis* challenge infection. Using this serum a parasite molecule of Mr 40kDa was discovered (*B. canis* antigen 1; BCA1). Partial amino acid sequences were determined. The genome of *B. canis* was sequenced and searched for DNA sequences that encoded for the three oligopeptides that had been identified. Results showed that the three peptide sequences were all encoded for by a single ORF. The gene was cloned and expressed in *E. coli* as a recombinant protein. Vaccination-challenge studies in dogs showed that the antigen induced protection against virulent *B. canis* challenge infection. Protection was reflected in decreased parasitaemia. BCA1 is GPI-anchored at the merozoite surface.

Comparative genomics and transcriptomics of Australian *Babesia bovis* strains

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We carried out Illumina sequencing to obtain the genomes and transcriptomes of three *Babesia bovis* strains from infected cattle in Australia. The parasites included (1) a hypervirulent field strain, (2) the strain that is currently used in the Australian live attenuated vaccine and (3) the original unattenuated field-derived parasite from which the vaccine strain was derived. *De novo* assembly and annotation of these genomes and RNA-Seq analyses of the sequenced mRNAs has allowed us, for the first time, to carry out a comparison of the genomes and transcriptomes of virulent and avirulent *B. bovis* parasites in Australia and to answer specific questions as to how *Babesia* parasites cause disease. With this approach, we will be able to identify the key virulence factors of *B. bovis* parasites and importantly elucidate the mechanisms that underpin the attenuation of a virulent strain.

Our comparative genomic analyses of virulent and avirulent strains has so far revealed 20 genes that are present in the virulent/unattenuated isolates but absent in the attenuated vaccine strain; strongly implicating their involvement in the virulence and pathogenicity of *Babesia* parasites. We are currently carrying out RNA-Seq analysis of the three parasite strains which will provide, for the first time, the complete transcriptome of *B. bovis*. This analysis is of paramount importance and will ensure that potential virulence factors are not overlooked by assuming that differences in virulence reside only at the genomic level. Analysis of the transcriptomic data may result in additional candidates being added to our list of likely virulence-related genes. Since there is no current transcriptomic data for any *Babesia* parasite, this will be the first time that such an analysis will be possible.

Identification and characterisation of exported *Babesia bovis* proteins

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Babesia bovis is the causative agent of bovine babesiosis, a disease of considerable economic significance to the livestock industry worldwide. The precise mechanisms by which this parasite causes disease in susceptible cattle are not well understood. It is clear, however, that pathophysiologically important alterations to the structure and function of red blood cells (RBCs) in which the parasites reside are secondary to the export of numerous, currently uncharacterised parasite-encoded proteins. Using a rational bioinformatic approach, we have identified a set of novel exported proteins in *B. bovis* that we believe are likely to be involved in alteration of infected RBCs and therefore highly likely to play major roles in the pathogenesis of babesiosis. We have identified at least 362 proteins in this subset, that also contain previously described exported proteins including merozoite surface antigens (MSAs), spherical body proteins (SBPs) VESA's and smorfs in addition to approximately 117 hypothetical proteins that currently have no identity to any other protein in any other organism. To characterise these unknown hypothetical exported proteins, we have developed a reliable transfection system to epitope tag or knockout genes that encode these proteins in *B. bovis*. Immunofluorescence analysis using specific antibodies raised against 2 of these proteins tested so far have confirmed their export and localisation in the infected RBC and this has been further substantiated by deletion and epitope tagging of the endogenous genes. Immunoprecipitation and pull-down assays using these newly created transgenic parasite lines is now being performed to identify interacting partners for these novel proteins and will help elucidate their function in the RBC. Further characterisation of these proteins in *Babesia* parasites as well as functional studies of transgenic parasite lines will assist in the longer term in identification of new and urgently required therapeutics for babesiosis.

RNA interference-mediated calreticulin silencing in *Babesia bigemina* infected tick *Rhipicephalus* (*Boophilus*) sp.

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Ticks are obligate hematophagous ectoparasites of wild and domestic animals as well as humans, considered to be second worldwide to mosquitoes as vectors of human diseases, but the most important vectors of pathogen-borne diseases of animals.

Babesiosis is one of the most important diseases vectored by ticks with a world-wide distribution affecting many species of mammals with a major impact on cattle. Particularity, *B. bovis* and *B. bigemina* are transmitted by cattle ticks, *Rhipicephalus (Boophilus) annulatus* and *R. microplus* being considered the most important cattle ectoparasites.

Calreticulin (CRT) has been identified in many different organisms and is implicated in diverse cellular processes including signaling, regulation of gene expression, wound healing, removal of cancer cells and autoimmunity. CRT is a protein that exists in ticks salivary glands and saliva probably related with ticks feeding and pathogen transmission, through its anti-thrombotic and complement inhibition functions. Previous studies reported that the expression of this protein is up regulated in *Babesia* spp. infected ticks indicating the possibility of using CRT as a recombinant vaccine against ticks and tick-bourne diseases.

In this study we analyzed the knockdown effect of CRT by RNA interference (RNAi) in both *R. annulatus* ticks. CRT double stranded RNA (dsRNA) was synthetized using specific primers and assays were conducted in groups of 30 adult female ticks of both *R. annulatus* and *R. microplus*. Negative and positive controls were used. Real-time quantitative PCR assays showed that knockdown of CRT reduced *B. bigemina* infection levels when compared to controls, in *R. microplus* ticks. Physiological parameters like survival rate and weight were also evaluated.

The results reported here increased our understanding on the role of tick genes in *Babesia* sp. infection and transmission.

Biodiversity and population genetics

Contrasting diversity and evolution of *Eimeria* and *Cryptosporidium*

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Vertebrate hosts susceptible to *Cryptosporidium* and *Eimeria* are phylogenetically broad. Many identifications of *Cryptosporidium* in vertebrates were prior to the use of molecular methods, and hence for many of the described hosts, *Cryptosporidium* can only be identified to genus. For *Eimeria*, species descriptions in over 900 vertebrate species are based on morphological characters. Molecular approaches are also unraveling complexity of species with the genus *Eimeria*. Information on the complex evolutionary pathways of these two apicomplexans is also being discovered through molecular analyses. Although we are gaining a greater understanding of the diversity and evolution of these two genera, we have only just touched the surface. Much of the molecular data has been obtained from limited hosts with a bias to those that are economically important. In addition to limited host range, samples are often from a single host representative and of narrow geographic range. These limitations mask the extent of parasite diversity and lead to biases in phylogenetic inference. In this presentation I will discuss the current status of diversity within *Cryptosporidium* and *Eimeria*, approaches to overcome sampling bias, and applications of emerging technologies to unravel the complex diversity within these two Apicomplexa genera.

Eimeria in the field - genetic diversity and population structure

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The chicken is the most numerous livestock species in the world. Infectious diseases that undermine efficient chicken production impact on local and global food security. Coccidioisis, the most economically significant parasitic disease of poultry, is caused by the apicomplexan *Eimeria* species. Control of these parasites has largely been based upon chemotherapy or live vaccination. Genes underlying susceptibility to immune or chemical killing have obvious relevance to the development of novel anticoccidial control strategies, but their identification has been demanding. Nonetheless, after decades of research a portfolio of realistic anticoccidial vaccine candidates has now been assembled. Laboratory studies using pure reference isolates and specific pathogen free chickens have identified Apical Membrane Antigen-1 (AMA-1), Immune Mapped Protein-1 (IMP-1) and Microneme Protein 3 (MIC3) as leading vaccine candidates. Now, building on published experiences with parasites such as the *Plasmodium* species, the efficacy and longevity of recombinant anticoccidial vaccines in the field using these and other antigens will rely on our understanding of naturally occurring allelic diversity and parasite population structure.

Genetic characterisation of the hybrid progeny of a cross between antigenically distinct *Eimeria maxima* strains before and after parental strain-specific immune selection has identified likely epitopic sequences for EmAMA-1 and EmIMP-1. Allelic sequence characterisation of a panel of historic and recent field isolates from more than 25 countries representing Asia, North America, South America, Africa, Europe and Australia has revealed distinct, but limited coding polymorphism within field parasite populations. These, and other sequences predicted to experience more neutral selection, are being combined in a multi-locus sequence typing (MLST) programme to infer population structure for *E. maxima* and *Eimeria tenella* and assess capacity for the development and spread of resistance against novel recombinant vaccines.

Subtypes and virulence of *Cryptosporidium parvum* in Germany

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Cryptosporidium is a protozoan parasite that causes enteritis, associated with aqueous, yellow or bloody diarrhea, dehydration, weight loss, fever and a mortality of 5-10% in calves.
The aim of this study was to characterize *Cryptosporidium parvum* isolates from Germany by subgenotyping and to determine in a cell culture assay whether different sub-genotypes respectively field strains have different degrees of virulence.
Faecal samples and epidemiological data from 478 calves of 99 dairy farms from all German federal states were analyzed. 282 (59%) specimens from 89 farms contained *Cryptosporidium* spp. 236 samples were identified as *Cryptosporidium parvum* and successfully subtyped. A total of 12 subgenotypes all belonging to the common allele family IIa were found with subtype IIaA15G2R1 being the most common (70%). In most of the farms we detected only a single subgenotype whereas in 7 farms we found multiple subgenotypes.
To unravel whether co-infections play a role in pathogenesis or not samples were regularly checked for viral and bacterial infections. 18% of all samples were positive for *Rotavirus*, 21% for *Coronavirus* and 10% for both, *Rota- and Coronavirus*. In only 2% of the samples *E.coli K99* was present. 52% of the animals were co-infected with *C. parvum* and one of the other pathogens.
To test for potential differences in virulence of the different field strains, oocysts were isolated from feces and used to infect an established HCT-8 cell line. To determine the detrimental effects of *C. parvum* on HCT-8 cells, a modified cell vitality test, based on methylthiazolyldiphenyl-tetrazolium bromide (MTT), was carried out. Most of the field strains did not show high differences in cell cytotoxicity in comparison to the in house strain. However, some of the strains displayed a 20% higher or lower cytotoxic effect indicating that virulence might differ between various stains.

Evidence of the three main clonal *Toxoplasma gondii* lineages in British wild carnivores

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Toxoplasma gondii is a zoonotic pathogen that has the ability to infect all warm blooded mammals including humans. Wildlife can act as reservoirs for *T. gondii* infection. Studying the genotype of *T. gondii* in wildlife provides an indication of strains which can potentially be transmitted to livestock and humans. Three main clonal lineages exist (type I, II and III) and within Europe type II is most commonly identified in humans and animals. Currently very little information exists relating to strains in Britain.

DNA was extracted from tissue samples from ferrets, polecats, badgers, foxes, mink and stoats, which had originated from various locations throughout the UK. A PCR, specific for *T. gondii*, was used to detect the presence of the parasite DNA, this was followed by strain genotyping (PCR-RFLP) and sequence analysis.

The prevalence of *T. gondii* within these animals varied from 6% to 44% depending on host species. Type II was the predominant lineage found, however type III and two alleles for type I were also identified, though no atypical genotypes were found.

The influence of genotype on human infection is still not fully understood, however certain genetic types may be associated with human clinical toxoplasmosis. This study highlights the presence of alleles for all three lineages with potential for their transmission to humans via infected livestock, or directly by cats.

Genetic diversity and geographic population structure of bovine *Neospora caninum* determined by microsatellite genotyping analysis

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The cyst-forming protozoan parasite *Neospora caninum* is currently recognised as one of the main causes of bovine abortion worldwide and is of great economic importance to the cattle industry. Recent studies have revealed extensive genetic variation among *N. caninum* isolates based on microsatellite sequences (MSs). These MSs could constitute suitable molecular markers for inferring the diversity of parasite populations, molecular epidemiology and the basis for phenotypic variations in *N. caninum,* which are currently poorly defined. In this work, we evaluated nine MS markers in a panel of 11 *N. caninum* reference isolates from around the world and 98 *N. caninum* bovine clinical samples and one ovine clinical sample collected from four countries on two continents -Spain, Argentina, Germany and Scotland - over a 10-year period of time. These markers were used as a molecular tool to investigate the genetic diversity, geographic distribution and population structure of *N. caninum*. Multilocus microsatellite genotyping based on 7 loci demonstrated very high levels of genetic diversity in the populations from all countries, with 96 microsatellite multilocus genotypes (MLGs) identified among 108 *N. caninum* samples. Geographical sub-structuring was present among the country populations according to the *F*-statistics and principal component analysis (PCA). The close genetic relationship observed between the Spanish and Argentinean populations is likely the result of parasite migration (the introduction of novel MLGs from Europe to South America) due to cattle movement. Finally, STRUCTURE clustering and Neighbour Net-work analyses showed the *N. caninum* is segregated into at least three groups that are genetically well differentiated as confirmed by PCA analysis. These groups were partially associated with geographic origin.

Invasion and motility

The rhoptry proteome of *Eimeria tenella* sporozoites

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Proteins derived from the rhoptry secretory organelles are crucial for the invasion and survival of apicomplexan parasites within host cells. The rhoptries are club-shaped organelles that contain two distinct subpopulations of proteins that localise to separate compartments of the organelle. Proteins from the neck region (rhoptry neck proteins, RON) are secreted early in invasion and are critical for the formation and function of the moving junction between parasite and host membranes. Proteins from the bulb compartment (rhoptry protein, ROP) are released later either into the nascent parasitophorous vacuole where they have a role in modifying the vacuolar environment and also into the host cell where they act as key determinants of virulence through their ability to interact with host cell signalling pathways, causing an array of downstream effects. In this paper we present the results of an extensive proteomics analysis of the rhoptry organelles from the coccidian parasite *Eimeria tenella*, which is a highly pathogenic parasite of the domestic chicken causing severe caecal coccidiosis. We have identified several different classes of rhoptry protein. First are the RON proteins, that have varying degrees of similarity to proteins of *Toxoplasma gondii* and *Neospora caninum.* We show that for some RON families, *E. tenella* expresses more than one gene product and that many of the individual RON proteins are differentially expressed between the sporozoite and merozoite developmental stages. We also show that the *E. tenella* sporozoite rhoptry expresses only a limited repertoire of proteins with homology to known ROP proteins from other coccidia, including just two secreted ROP kinases, both of which appear to be equipped for catalytic activity. Finally, we identify a large number of hitherto undescribed proteins that map to the sporozoite rhoptry many of which have orthologous proteins encoded within the genomes of *Toxoplasma gondii* and *Neospora caninum*.

Is gliding motility essential for invasion?

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It is believed that apicomplexan parasites actively invade their host cell which depends on an arsenal of secreted invasion factors, such as the thrombospondin related proteins (TRAP, MIC2) and the actin myosin system of the parasite. During invasion MIC2 interacts with actin via the glycolytic enzyme aldolase and is translocated to the posterior pole of the parasite which provides the necessary force to push the parasite into the host cell via the moving junction. This model is well supported by the analysis of conditional knockdown mutants for MyoA, MIC2 and actin interacting proteins and suggest an essential function of this invasion machinery in *Toxoplasma gondii* and *Plasmodium*. However, a complete block in host cell invasion cannot be achieved in these mutants and this discrepancy has been explained by background expression of the respective protein in the knockdown. To address this discrepancy, we generated a novel conditional recombination system based on dimerizable Cre-recombinase (DiCre), and we employed this system to generate conditional knockout mutants for MIC2, MyoA and actin. We show that MyoA and MIC2 are not as previously believed essential for the parasite *in vitro* and that parasites lacking these factors are well capable of invading the host cell. Furthermore, we found a novel function for parasite actin for the replication of the apicoplast, a platid-like organelle in apicomplexans. Intriguingly, parasites lacking actin are still capable of invading the host cell, demonstrating that parasites are capable of invading the host cell in an actin-myosin independent manner.

A molecular basis for the restricted host and tissue tropism of *Eimeria* parasites

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The phylum Apicomplexa is home to a wide variety of parasites of significant medical and economic relevance, including the coccidian species *Toxoplasma gondii* and *Eimeria tenella*. These parasites have extremely different host and tissue tropisms; *T. gondii* can invade virtually any nucleated cell and infect almost all warm-blooded vertebrates, whereas *E. tenella* infects only chickens and is restricted in its growth to epithelial cells of the caecum.

Proteins (MICs), released from the microneme secretory organelles, are important for apicomplexan invasion of host cells and many MICs bear modular arrangements of sequences with homology to adhesive proteins from higher eukaryotes. It has been shown for *T. gondii* that some MICs are absolutely critical (essential) for invasion, whereas others are not essential *per se* but are nevertheless extremely important because they allow the parasite to bind a diverse range of host cell oligosaccharide epitopes. Two families of MICs, the sialic-acid binding MAR-domain containing proteins (MCPs), and the galactose-binding Apple-domain containing proteins (ACPs) are suggested to make significant contributions to different host and tissue tropisms of *T. gondii* and *E. tenella*. In this paper, we present new information on MCPs and ACPs of *E. tenella* and discuss this in the context of what is currently known about the structural basis of the interaction of these domains with carbohydrate moieties.

Immunolocalisation dynamics of NcROP40, NcROP2, NcGRA7 and NcNTPase throughout the lytic cycle of *Neospora caninum* tachyzoites

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Proteins associated with the surface, micronemes, rhoptries, and dense granules are essential elements during the intracellular lytic cycle of apicomplexan parasites such as *Neospora caninum*. Due to their role in host parasite dissemination, these elements could constitute potential drug targets and vaccine candidates. The dense-granule protein NcGRA7 has been described as an immunodominant protein, whereas the rhoptry protein NcROP2 has been tested as vaccine candidate against *N. caninum* infection in mice. In addition, higher expression levels of the rhoptry protein NcROP40 and the dense-granule protein NcNTPase were detected in virulent *N. caninum* isolates by proteomic approaches. Several rhoptry proteins have been recognised as virulence factors, and NTPase has also been shown to be associated with virulence in the related parasite *Toxoplasma gondii*. To perform the functional characterisation of these proteins, an *in vitro* immunolocation time-course study using confocal laser-scanning microscopy was carried out throughout the tachyzoite lytic cycle during the adhesion-invasion, proliferation and egress phases. Specific polyclonal antibodies were raised in rabbits inoculated with recombinant NcROP2, NcROP40, NcGRA7 and NcNTPase proteins that were produced in *Escherichia coli* and purified by affinity chromatography.
Immunocytochemistry confirmed the predominant localization of NcROP2 and NcROP40 in rhoptries and of NcGRA7 and NcNTPase in dense granules. Notably, in contrast to other soluble rhoptry proteins, NcROP40 is maintained in the rhoptries during the entire lytic cycle and does not appear to be secreted, whereas NcROP2 was released starting in the early stages of the lytic cycle until the exponential proliferation of the parasite. NcNTPase presented a more diffuse pattern during the early stages than during exponential growth, during which time it was localised to the dense granules, although its presence in parasitophorous vacuole lumen was not discerned. NcGRA7 was secreted into the parasitophorous vacuole during parasite multiplication, as previously described. This is the first work that immunolocalised the NcROP40 and NcNTPase proteins, although further studies must be carried out to clarify the functional roles of these proteins.

The role of *Cryptosporidium parvum* calcium dependent kinase 1 (CDPK1) in the invasion of host cells

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Cryptosporidia are Apicomplexa that have lost their apicoplast but retained some plastid derived genes which are believed to play an important role in pathology. Among them are the so called calcium dependent protein kinases (CDPK) which are involved in signal transduction. For *Toxoplasma* and *Plasmodium* it is shown that CDPKs play an important role in the invasion into and the egress from the host cell by triggering the release of the microneme content leading to the formation of the parasitophorous vacuole (PV). This makes it highly attractive to study CDPKs for two reasons. First, they will give us more insight into the cell-parasite interactions on a molecular level. Secondly, since CDPKs are essential enzymes in Apicomplexa with no counterparts in the mammalian host they are promising drug targets.
Using an HCT-8 infection model, the expression of all seven *C. parvum* CDPKs at different time points was analyzed by 3'-RACE-PCR revealing different expression profiles. CpCDPK1, the orthologue of *Toxoplasma gondii* CDPK1 that is shown to be important in microneme secretion, showed an expression pattern that corresponds to the invasion events of *C. parvum*. To confirm these findings an mAb was raised against CDPK1 and used to detect CDPK1 expression through Western blot and IFAT. The results of the Western blots showed that the antibody reacted with a 56 kDa protein, corresponding very well to the predicted 55,72 kDa. Analysis of the expression pattern through qPCR revealed a strong upregulation of CDPK1 expression at 18-20 h p.i. and a minor upregulation at 50 h p.i. This points to a link between the expression pattern of CDPK1 and the invasion events of *C. parvum.* Therefore CDPK1 is an attractive candidate for further studies of the correlation between parasite calcium signaling and infection of the host cell by *C. parvum*.

Intracellular survival and host-parasite relationship

Central carbon metabolism in Apicomplexa: versatility and adaptation to an intracellular life style

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Plasmodium species and *Toxoplasma gondii* possess a complete tricarboxylic acid (TCA) cycle to generate precursors for ATP synthesis and provides carbon rich intermediates for key anabolic pathways. However, the absence of mitochondrial pyruvate dehydrogenase (PDH) failed to establish a link between glycolysis and mitochondrial carboxylic acid metabolism and posed a dilemma regarding the source(s) of acetyl-CoA to fuel the canonical oxidative TCA cycle. A solution to this problem came with the report of a branched TCA cycle in the human malaria parasites that utilize glutamine and selectively develops in erythrocytes. Alternatively, we previously postulated that the mitochondrial branched chain keto-acid dehydrogenase (BCKDH) could substitute for the absence of PDH. BCKDH is present even in *Plasmodium sp*. that lack other enzymes of the branched chain amino acid degradation pathway. Gene deletion of E1α subunit in *T. gondii* and *Plasmodium berghei* establishes that BCKDH significantly contributes to growth *in vitro* and virulence *in vivo*. Metabolic profiling by LC-MS and GC-MS combined to metabolic flux analysis using U-^{13}C labelled carbon sources provided evidence that the BCKDH possesses a PDH-like activity to convert glycolytic pyruvate to mitochondrial acetyl-CoA to sustains a complete TCA cycle in both parasites. Deletion of TgE1α subunit leads to accumulation of pyruvate, a dramatic drop in acetyl-CoA, activation of gluconeogenesis and an alteration of apicoplast FAS II biosynthesis. The absence of PbE1α hinders parasite development within mature erythrocytes in the mouse model, leading to a severe anaemia despite a low parasitaemia limited to reticulocytes. Collectively, these results suggest that both parasites catabolize glucose via the TCA cycle, and that this cycle is essential for optimal growth and for virulence.

Toxoplasma gondii and subversion of its host cell epigenome: the price to pay to survive

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Posttranslational modifications of proteins represent efficient strategies to modify activities, halflives, or the intracellular localization of host proteins that are critical for infection. *Toxoplasma gondii* secreted several rhoptry bulb resident kinases and phosphatases that co-opt host cells by interfacing with their signalling pathways and thereby controlling the outcome of the infection. Although multiple phenotypes have been dressed during *Toxoplasma* infection, few effector proteins have been characterized so far. GRA22 was identified in the course of our initial genetic screen that reveals novel secreted proteins and evidence for non-classical GRA protein secretion. GRA22 is one of the rare GRA proteins that is abundantly exported to the host-cell nucleus during infection. The protein is embedded in multiple high-molecular weight complexes with host proteins such as p300/CBP remodelers or the MAPK p38. In line with previous studies, we report an unexpected activation mechanism for p38 MAPK following *Toxoplasma* infection that does not involve the prototypic kinase cascade. Rather it depends on interaction of p38 with GRA22 leading to autophosphorylation and activation of the host kinase, regardless of the strain type. GRA22-dependent p38 MAPK activation culminates in the induction of multiple proinflammatory cytokines expression, including IL-12p40. While eliciting a classical M1 macrophage activation, GRA22 was also able to strongly repress M2 response genes (e.g. *Arg1*) in type II strain, suggesting that the protein is an essential regulator of macrophage M1/M2 polarization and attendant functions. Taken together, our findings identify a new alternative MAPK activation pathway important in modulating host cell responses to *Toxoplasma* infection.

Recruitment of microtubules by the intracellular parasite *Theileria*: characterization of an EB1-binding parasite surface protein

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The strictly intracellular Apicomplexan parasite *Theileria* is unique in its ability to transform the infected host cell, inducing uncontrolled proliferation and conferring resistance to apoptosis. This poses an interesting conundrum; how does this large parasite ensure its persistence in the cytoplasm of a continuously dividing cell? We have previously shown that *T. annulata* associates with the host cell central spindle in a Plk1 dependent manner, and that this association, together with an interaction with astral microtubules, is crucial for the faithful segregation of the parasite between two daughter cells. We now demonstrate that the plus end tracking protein (+TIP) EB1, the core component of +TIP networks, interacts with the parasite, most strikingly as the cell exits mitosis. We have identified a *T. annulata* surface protein that interacts with EB1 via an EB1-binding "SxIP" motif. This is the first parasite encoded "EB1-binding protein" to be characterized, and we show that it is phosphorylated in a cell cycle dependent manner, and tracks growing microtubule plus ends when expressed in COS7 cells. Targeting of the parasite protein to the mitochondria membrane of COS7 cells resulted in the recruitment of endogenous EB1 to the mitochondria, confirming the interaction between these two proteins.

Protozoan induced direct activation of bovine NK cells is inhibited by soluble antigens from *Toxoplasma gondii* and *Neospora caninum*

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Natural Killer (NK) cells play a key role in the early innate immune responses during protozoan infections. Activation of NK cell by the intracellular protozoan *Toxoplasma gondii* is accessory cell dependent, however we have previously shown that tachyzoites from the closely related *Neospora caninum* triggered bovine NK cells to produce gamma interferon (IFN-γ) directly. In the present study, we compared the IFN-γ responses of purified bovine NK cells to various antigen preparations from *T. gondii* and *N. caninum*. These antigen preparations consisted of live and heat-inactivated tachyzoites, sonicated tachyzoites, the soluble and insoluble fractions of the sonicated parasites, and tachyzoite secretory fractions. The activating substances from *N. caninum* were present in the membrane lipoprotein fraction that was extracted from the insoluble fraction of the sonicated tachyzoites. This indicated that the direct stimulation of NK cells was not dependent on an intact tachyzoite surface. In contrast, antigens prepared from *T. gondii* did not trigger IFN-γ production from bovine NK cells. Furthermore, soluble antigens from *T. gondii* and *N. caninum* inhibited the parasite-induced IFN-γ response from NK cells. These findings suggest that NK cells are both directly activated and inhibited by different antigen preparations from protozoan parasites however the mechanisms lying behind this processes remains to be elucidated.

Mice congenitally infected with low-to-moderate virulence *Neospora caninum* isolates exhibited clinical reactivation during the mating period without transmission to the next generation

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Endogenous transplacental transmission (EnTT) is the major transmission route of *N. caninum* in cattle. Thus, the development of an appropriate experimental model of EnTT is needed for more appropriate analysis of the parasite biology and control strategies. A recent study reported EnTT rates of up to 40-50% in chronically infected dams with low-to-moderate and high virulence *N. caninum* isolates. In the present study, low-to-moderate virulence *N. caninum* isolates (Nc-Spain 3H; G1 and Nc-Spain 8; G2) that previously showed high TT rates but low mortality and morbidity rates in a congenital mouse model were inoculated to dams (first generation). The new approach followed in the present study aimed to start with a high number of congenitally infected mice (second generation) to allow more efficient EnTT from congenitally infected dams to their progeny (third generation). Interestingly, reactivation of the infection occurred in several congenitally infected non-pregnant females (second generation) in both infected groups, as evidenced by neosporosis-associated clinical signs between induction of the Whitten effect and mating, accompanied by an increase in specific antibodies levels (IgG1, IgG2a and anti-rNcGRA7) ($P< 0.0001$; one-way ANOVA) and a higher number of PCR-positive mice relative to the number of PCR-positive pregnant females ($P<0.05$; Fisher's exact test). These results support the hypothesis that only mice without clinical signs and with a low parasite burden in the brain can become pregnant, which may explain the inability to induce EnTT between the second and third generations. However, interestingly, clinical reactivation was successfully induced in congenitally infected mice. These findings confirm that this mouse model is not a suitable experimental EnTT model for testing the efficacy of drugs and vaccine candidates against EnTT. The employment of other suitable species with a similar placenta structure, such as small ruminants, should be considered.

Comparison of the maternal and foetal immune responses of cattle, following an experimental inoculation with *Neospora caninum* at early, mid and late gestation

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The following experiments describe the maternal and foetal immune responses in cattle following a subcutaneous inoculation with live *Neospora caninum* tachyzoites at 70, 140 and 210 days of gestation (dg). At each stage of pregnancy a serial analysis of the maternal and foetal local and peripheral immune responses was conducted at 14, 28, 42 and 56 days post inoculation (dpi).

At 70dg, foetal deaths were recorded from 28dpi onwards, challenged dams carrying live foetuses showed consistently higher levels of cellular proliferation and significantly ($p \leq 0.05$) higher levels of IFN-γ compared to those carrying dead foetuses. Samples of foetal spleen, thymus and PBMC demonstrated cellular proliferation as well as IFN-γ, IL-4, IL-10 and IL-12 production following mitogenic stimulation with Con A from 14dpi (day 84 gestation) onwards.

At 140dg, PBMC from all challenged dams demonstrated antigen specific proliferation and IFN-γ production by 7dpi. Challenged dams seroconverted producing *Neospora*-specific IgG from 7-14dpi, while anti-*Neospora* antibodies were detected in the foetuses from 42dpi. Maternal and foetal *Neospora*-specific CMI responses were demonstrated in local and peripheral lymph nodes by 14dpi and 28dpi respectively. On 42dpi phenotypic analysis of the responding cells showed that they were predominantly CD4+ T-cells, in both dams and foetuses.

At 210dg, seroconvertion of the dams occurred between 7-14dpi, while significantly ($p \leq 0.05$) increased levels of cellular proliferation and production of IFN-γ were seen in both the maternal and foetal local and peripheral lymph nodes of the challenged animals from 14dpi, along with increased levels of IL-4 and IL-10. From 28dpi significant ($p \leq 0.05$) increases in expression of TLR-2 and TLR-9 were observed in challenged dams, while increased levels of both TLR-2 and TLR-9 were seen in the foetuses of challenged dams.

Our results show that robust maternal and foetal immune responses are required to ensure foetal survival following a challenge with *N. caninum.*

Concomitant parameters promoting *Neospora*-induced abortion in cattle?

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Since the eradication of BVD, *Neospora caninum* is definitely the most important infectious cause of bovine abortion in Switzerland. Vertical transmission accounts for at least 90% of all *N. caninum* infection cases. This clearly indicates that in a chronically infected but apparently healthy bovine animal, the infectious agent remains viable putatively lifelong. Vertical transmission conventionally occurs during gestation. Various reports indicated so far that hormoneal alterations during gestation may trigger reactivation of the parasite and thus lead to subsequent vertical (transplacental) infection. As the consequence of reactivation nevertheless considerably varies in respect to the outcome from abortion to non-symptomatic transmission to the fetus, the question arises, if additional factors contribute causatively to abortion.

Our respective working hypothesis is based on two main subjects putatively down-regulating transient immunity of the dam: (a) *N. caninum* can be additionally activated during pregancy by mycotoxins, based upon either a hormone-like effect, or, more likely, by inducing transient co-immunosuppression in the (pregnant) host animal; (b) additional immunological stress of the dam such as induced through vaccination, thus resulting in a vaccine-induced *Neospora*-associated abortion.

Currently, we are addressing both subjects upon appropriate field studies:

In study (a), we focus our investigations on the the mycotoxin “mycophenolacid”, frequently produced by *Penicillium roqueforti* growing often in moulded fodder; this agent is known as being extremely immunosuppressive. A case-control study will elucidate this parameter, by means of determination of the concentration of mycophenolacid in food samples obtained from the individual farms, respectively, in association to *Neospora*-associated abortion problems.

In part (b), we address experimentally if an association exists between reactivation of a latent *N. caninum* infection and a concomitant vaccination, i.e. BTV vaccination, which would thus explain the higher-than-expected incidence of *N. caninum* abortion that has been descriptively observed after BT vaccination in Switzerland in 2010.

Diagnosis

Bovine besnoitiosis: Antibody detection in diagnosis and in epidemiological studies

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Bovine besnoitiosis – caused by the protozoan *Besnoitia besnoiti* – is a severe disease with significant economic impact in Africa, Asia and Europe and has been re-emerging in several parts of Europe recently (http://www.efsa.europa.eu/en/scdocs/scdoc/1499.htm). Introduction of infected cattle into naive herds seems to play a major role in the transmission of the infection among herds. Therefore, early detection of infection is essential to prevent the spread of bovine besnoitiosis.

A number of serological techniques, including IFAT, ELISA and immunoblots have been developed for the diagnosis of besnoitiosis. A recent multi-centred study (*1*) showed that the sensitivity of serological tests was low in cases of early *B. besnoiti* infection. In addition, some tests had reduced sensitivity in animals sampled after a climatic season with low or no insect activity, i.e. after phases in which mechanical transmission by insects was low or even absent (*2, 3*). Diagnostic problems result also from suboptimal specificity, which may cause a high proportion of false-positive reactions (*4*), and protocols are needed to improve the specificity of antigen preparations used for serological diagnosis.

Serological techniques are important tools for epidemiological studies. During experiments to elucidate the life cycle of *B. besnoiti*, the antibody responses of potential intermediate and definitive hosts were examined (*5*). Testing the avidity of the *B. besnoiti*-specific IgG response (*6*) may allow to define the state of infection of individual animals in a herd and to assess the time period when the infection was introduced or transmitted within a herd.

1. Garcia-Lunar et al., Transbound. Emerg. Dis., in press (2012), 2. Schares et al., Vet. Parasitol. 175, 52 (2011), 3. Lienard et al., Vet. Parasitol. 177, 20 (2011), 4. Nasir et al., Vet. Parasitol. 186, 480 (2012), 5. Basso et al., Vet. Parasitol. 178, 223 (2011), 6. Schares et al., Int. J. Parasitol., submitted (2012)

First cases of bovine besnoitiosis in Switzerland

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In Europe, bovine besnoitiosis occurs endemically in Portugal, Spain and France, and focal outbreaks have been reported in Germany and Italy in the last years. To determine if *Besnoitia besnoiti* has been introduced into Switzerland through the import of breeding cattle from France, a systematic serological survey was performed. A total of 412 breeding cattle from 114 farms, imported from France between 2005 and 2011, were serologically examined for antibodies against *B. besnoiti* using a commercial ELISA kit (PrioCHECK© Besnoitia Ab 2.0, Prionics AG, Zurich, Switzerland). Serum samples reacting positive in the ELISA were subsequently tested by an indirect immunfluorescence test (IFAT) and a Western blot (WB) using *B. besnoiti* tachyzoites antigens. The serologic diagnosis was confirmed by IFAT and WB in 2 Limousin cows imported from France on a farm in Eastern Switzerland. Subsequently, this whole herd (n=16) was examined clinically and serologically, and 2 additional Limousin cows imported from Germany also reacted positive in the three serological tests. One of these cows presented *B. besnoiti* tissue cysts in the scleral conjunctiva and typical skin lesions in the head region. The infection was further confirmed cytologically, histopathologically and by PCR. Further studies to evaluate a possible spreading of the parasite from this farm and the occurrence of more cases of bovine besnoitiosis in Switzerland are on-going.

New real time PCR to differentiate *Sarcocystis* spp. affecting cattle

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Cattle are intermediate hosts (IH) of different *Sarcocystis* spp.: *S. cruzi*, *S. hirsuta* and *S. hominis* which use canids, felids or primates as definitive hosts (DH), respectively. Infections produce mainly chronic thin-walled (*S. cruzi*) or thick-walled tissue cysts (*S. hominis* and *S. hirsuta*). Recently, *S. sinensis*, which was first described in China, has also been diagnosed in minced beef in Germany. The DH of *S. sinensis* is unknown. The aim of the present study was to develop and optimize new real-time 5'-nuclease quantitative PCR assays for a sensitive and specific diagnosis of *Sarcocystis* spp. in cattle and to develop molecular tools to differentiate *S. sinensis* from *S. hominis*.

Fragments of the 18S rDNA were amplified from individual sarcocysts and cloned to serve as controls in real-time PCR assays. Primers targeting a conserved region that flanks a variable region of the 18S rDNA were selected and individual probes for each *Sarcocystis* spp. designed. Efficiency of amplification by different primer concentrations and temperature protocols were analyzed. For the identification of *S. cruzi* and *S. hirsuta,* conventional probes were suitable, but for the differentiation of *S. hominis* and *S. sinensis* probes with Locked Nucleic Acids (LNA) modifications were necessary. Each assay was evaluated individually and subsequently combined in a multiplex assay. The analytical specificity of the multiplex assay was assessed using 5 ng of DNA of heterologous *Sarcocystis* spp., *Neospora caninum*, *Toxoplasma gondii*, *Hammondia* spp. and *Besnoitia besnoiti*. No positive reactions were observed using DNA of others apicomplexan parasites than the species the PCR targeted. The analytical sensitivity was estimated using dilutions of plasmid DNA specific for the individual species and DNA of isolated bradyzoites. It ranged between 2-20 DNA copies or 0.1-0.3 bradyzoites. This method allows the sensitive and specific identification of *Sarcocystis* spp. affecting cattle in single and mixed infections.

Is there a need for improved *Cryptosporidium* diagnostics in Swedish calves?

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Cryptosporidium analysis at the National Veterinary institute (SVA) only includes direct microscopy to determine presence of oocysts. Because *C. bovis* is a common species in Swedish preweaned calves, and we previously have identified *C. bovis* in diarrheal calf samples, we evaluated whether routine diagnostics need to include further analysis to assess pathogenicity of detected oocysts. Diarrheal calf samples sent for *Cryptosporidium* analysis to SVA from March 2010-March 2012 were used. Faecal consistency and colour were noted. A questionnaire regarding diarrhoeal problems was sent to the farmers. *Cryptosporidium* positive samples were concentrated by NaCl-flotation and oocysts were enumerated by epifluorescence microscopy. Molecular analysis of the ssrRNA and GP60 genes was performed. 782 samples were sent for *Cryptosporidium* analysis. 198 of these were *Cryptosporidium* positive. Oocyst counts were 100 to $55x10^6$ OPG. *C. parvum* was identified in 178 samples, *C. bovis* in 6 samples and mixed *C. parvum/C. bovis* in 7 samples. *C. parvum* infection was most common at 1-3 weeks of age, whereas *C. bovis* positive calves were older. Oocyst counts were higher for *C. parvum*. 27 *C parvum* subtypes were identified. With three exceptions, only one subtype per herd was identified. Subtype family (SF) IIa was most prevalent and was associated with watery faeces compared to SF IId, and there was a tendency towards higher oocyst counts and need for more rehydration for SF IIa.
We conclude that there is no need to evaluate *Cryptosporidium* at species level for diarrhoeal samples from Swedish herds with ongoing problems because these calves do not reflect the average species distribution in preweaned calves. The dominance of *C. parvum*, especially in the period when clinical cryptosporidiosis is most common, and higher oocyst counts support that this species is more pathogenic than *C. bovis*. The possible pathogenic potential of *C. bovis* warrants further attention.

Improvement of immunohistochemical diagnosis of *Neospora caninum* using monoclonal antibodies

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Neospora caninum is a major cause of bovine abortion worldwide. Several diagnostic techniques have been employed to identify infected animals, but the disease is only confirmed by histopathologic examination followed by immunohistochemical (IHC) or molecular tests. The aim of this study was to test the suitability of monoclonal antibodies (mAb) in the IHC for *N. caninum*. We hypothesized that using a cocktail of mAb against different *N. caninum* epitopes will result in an IHC test with high specificity and sensitivity. Three mAb to *N. caninum* (4.15.15, 4.11.5 and 1/24-12) were used alone or in combination as primary antibodies in IHC and compared with polyclonal antibody (pAb). Positive controls consisted of sections of bovine brain mixed with cultured *N. caninum* tachyzoites (for quantitative purposes), and bovine tissues from naturally-infected animals. Four mAb combinations and a pAb were tested for their reactivity against *N. caninum* in tissue sections. Bovine tissues containing other cyst-forming coccidia (*Toxoplasma gondii*, *Besnoitia besnoiti* and *Sarcocystis* sp.) were included to confirm specificity. The mAb 4.15.15, 4.11.5 and 1/24-12 individually tested detected 57%, 49% and 41% of the total parasites in the sections, respectively. The four different mAb combinations used to quantify *N. caninum* in the bovine brain mixed with tachyzoites detected 32.4% up to 79.4% of the parasites. The best mAb combination was tested with different tissues from naturally infected cattle. The mAb combination and the pAb detected *N. caninum* in 100% (18/18) of tissue sections from naturally infected animals. *Sarcocystis spp.* or *B. besnoiti* were not detected using any mAb combination; however, pAb labeled *Sarcocystis* sp. cysts. We concluded that the cocktail of mAb used in our study had a good performance in the IHC for *N. caninum.*

Control strategies
(vaccination and chemotherapy)

Control of *Neospora caninum* and *Toxoplasma gondii* in farm livestock

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Neospora caninum and *Toxoplasma gondii* are important causes of reproductive failure and production losses in farm livestock worldwide. In addition, consumption of undercooked meat containing *T. gondii* cysts is an important route of transmission to people. *T. gondii* is currently recognized as one of the most important food borne pathogens with one in five people worldwide thought to be infected.

Our research approach has involved establishing experimental models of infection in livestock species to enable the study of the host parasite interaction in a relevant host. This has improved our understanding of how the parasites establish infection in the host and how the innate and adaptive immune responses are induced in response to the parasite and the outcomes of this critical interaction.

This paper will highlight and discuss how knowledge of the host-pathogen interaction in livestock species has helped in the development of disease prevention and control strategies for *N. caninum* and *T. gondii* involving biosecurity, diagnostics and vaccination approaches.

Vaccine development for bovine neosporosis: present situation and *in vitro* and *in vivo* models to test safety and efficacy

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Vaccination is considered the best option for the cost-effective control of bovine neosporosis. In pregnant animals, *Neospora caninum* reaches and crosses the placenta and infects the foetus, multiplying in different vital organs. Such foetal infections can lead to abortion, stillbirth, the birth of weak calves or, alternatively, the birth of clinically healthy but persistently infected calves that can transmit the pathogen to their progeny in the case of females. Consequently, an effective vaccine for bovine neosporosis must demonstrate protection against congenital transmission, and the two key parameters that must be evaluated are foetal death and vertical transmission. Several approaches have been assessed for the development of safe and effective vaccine products for bovine neosporosis. These approaches include killed whole-parasite vaccines, live-attenuated vaccines and sub-unit vaccines, among others. One key factor in testing the safety and efficacy of vaccines is the availability of standardised and accurate *in vitro* and *in vivo* models. During the 2011 meeting of the World Association for the Advancement of Veterinary Parasitology held in Buenos Aires, a workshop addressing "Perspectives for control for cattle reproductive diseases caused by protozoans" stressed the urgency of developing standardised pre-clinical and clinical models for *N. caninum*. These models are essential to study host-pathogen interactions, to investigate host immunity at the local and systemic level, and to evaluate vaccine candidates and therapeutics. In this presentation, the results obtained and the advantages and drawbacks of the different vaccine approaches will be summarised and discussed. In addition, *in vitro* and animal models used for vaccine testing will also be presented. The necessity of consensus guidelines, including the isolates/strains of *N. caninum* used, the challenge dose, the time and route of challenge, the preparation of the inoculum, the animal model (mice *versus* cattle; sheep *versus* cattle), and other parameters, will be discussed, and international standard guidelines that can gain acceptance worldwide will be proposed.

Efficacy and safety of vaccination in cattle with live tachyzoites of *Neospora caninum* for the prevention of *Neospora*-associated fetal loss

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Vaccination with live *N. caninum* protects against fetal death in challenge models of infection of cattle and mice, and the naturally attenuated NC-Nowra strain of *N. caninum* is of interest as a vaccine. NC-Nowra was originally isolated from a congenitally infected calf, sourced from NSW Australia, and subsequently shown to prevent transplacental transmission in mice and fetal death in cattle. In this study, a vaccinate and challenge model was used to investigate the effect of route of administration of NC-Nowra as a live vaccine. Vaccination of heifers prior to breeding with live NC-Nowra tachyzoites by either the subcutaneous or intravenous route reduced the rate of abortion and presence of the parasite in calves as determined by PCR and serology after challenge of cows with a virulent isolate.Leve ls of protection ranged from 55.6% to 85.2% depending on the route of vaccination and growth conditions of the vaccine strain used with cryopreserved tachyzoites being less effective (25.9% protection). This study confirms that live vaccination can be an effective method of preventing neosporosis in cattle, and highlights the technical hurdle of preservation of live parasites that must be overcome for a vaccine to be commercially successful.

Effect of vaccination of cattle with the naturally attenuated Nc-Spain 1H isolate of *Neospora caninum* on responses to heterologous challenge during early and mid gestation

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Live vaccines have emerged as one of the main options for the development of cost-effective measures for the control of bovine neosporosis. Previous studies have shown that Nc-Spain 1H is a naturally attenuated isolate of *Neospora caninum* and that immunisation using live tachyzoites generated a protective immune response in mice. The aim of this study was to evaluate the safety and efficacy of Nc-Spain 1 H when used as a vaccine isolate in cattle. *N. caninum*-seronegative heifers were immunised subcutaneously twice with 10^7 live Nc-Spain 1H tachyzoites at four-week intervals prior to artificial insemination. A group of animals was challenged intravenously with 10^7 live Nc-1 tachyzoites at 70 days of gestation, and another group was challenged with 4.4×10^8 live Nc-1 tachyzoites at 135 days of gestation. In the immunised/non-challenged group, no foetal death was observed; calves were negative for pre-colostral *Neospora* antibodies, and parasite DNA was not detected in either calves or their dams. In the group immunised and challenged during early gestation, 3 out of 5 foetuses survived to term and were born clinically normal, whereas foetal death occurred in 4 out of 5 the non-immunised/challenged heifers. In the group immunised and challenged at mid gestation, the calves showed significantly lower pre-colostral *Neospora*-specific antibody titres than calved from the non-immunised/challenge group. Strong antibody and interferon gamma responses were induced in the immunised heifers. This study confirmed the low virulence of the Nc-Spain 1H. The results indicated that this isolate was unable to establish a detectable persistent infection in cattle, indicating the safety of subcutaneous immunisation with live Nc-Spain 1H tachyzoites prior to mating. Moreover, this immunisation protocol reduced the occurrence of *N. caninum*-associated abortion and vertical transmission after highly aggressive heterologous challenge during early and mid gestation. The following step in the evaluation of this live vaccine candidate should be the assessment of its safety and ability to prevent abortion and vertical transmission in cattle under field conditions.

VitamFero: Novel proprietary live attenuated vaccines against Apicomplexa

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VitamFero is a young French biotechnology company developing a new generation of Apicomplexa vaccines against various parasitoses affecting farm animals. Thanks to its proprietary technology, its specific know-how and its growing vaccine platform, VitamFero, along with the François-Rabelais University of Tours and INRA, is able to develop safe, efficient, and easy-to-produce vaccines based on the engineering of live attenuated parasite strains obtained by total, controlled and irreversible deletions of specific genes of virulence. Strong proofs-of-concept have already been obtained in targeted species for several vaccine candidates currently in development, among them, toxoplasmosis, neosporosis and cryptosporidiosis of ruminants.

VitamFero's vaccine competitive advantages are supported by robust in-vivo data showing a higher efficacy rate than competitors, a better safety and regulatory profile than other live attenuated vaccine with no risk of return to virulence and an easier vaccination protocol with one single injection to reach a long-term protection with no boost required.

According to VitamFero's development plans involving business partnerships with big animal health firms, our first 2 vaccines are to be launched in 2015 followed by 4 others between 2016 and 2018.

Structure-aided design of calcium-dependent protein kinase inhibitors for selective drug treatment of *Apicomplexan* infectious diseases

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Effective treatment of infectious apicomplexan diseases is a formidable public heath challenge that will require new therapeutic strategies. Protein kinases are attractive drug targets for eukaryotic pathogens as key players in signal transduction, regulating important cellular processes including protein synthesis, gene expression, subcellular localization of proteins, host cell invasion, gliding motility, and microneme secretion. Calcium dependent protein kinases (CDPK) are especially promising because orthologs are absent in mammalian genomes. Unlike most mammalian kinases, many apicoplast CDPKs have small gatekeeper residues within their ATP binding site, which render *them* more sensitive to bumped kinase inhibitors (BKIs). Thus, a design of selective inhibitors with minimal off-target effects may be achievable. Apicoplast CDPK homologues have been expressed and purified from *Babesia bovis*, *Cryptosporidium parvum*, *Eimeria teneca*, *Neospora caninium*, *Plasmodium falciparum* and *Toxoplasma gondii*. We have designed a series of BKIs that specifically exploit the gatekeeper pocket and are using structure-based drug design approach to evolve them into specific anti-apicoplast CDPK leads that display desirable properties against the mammalian host. About 400 BKIs have been synthesized and tested for inhibition of Apicomplexan CDPKs, while confirming specificity over mammalian kinases with small gatekeeper residues including Src, Abl, and Hck. Crystal structures of many BKIs in complex with both *Tg*CDPK1 and Src have been solved and used in the design of inhibitors that are several orders of magnitude more selective for the parasite kinase over a panel of host kinases. Selected high quality compounds have been tested for drug lead potential by measuring toxicity against apicoplast cells relative to several mammalian cell lines and some of these have been evaluated for pharmacokinetic (PK) properties in a mouse model. Our studies thus far indicate that this strategy leads to non-toxic, selective inhibitors with good PK properties, and are thus excellent leads for further drug development.

Chlorothiazolides as effective anticryptosporidial agents *in vitro* and in immunosuppressed gerbils

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Nitazoxanide (ntz) is a broad-spectrum anti-infective drug that adversely affects growth and proliferation of extracellular and intracellular protozoan parasites. Non nitro-thiazolides and particularly chlorothiazolides have been shown to be effective *in vitro* against the apicomplexan parasites *Neospora caninum*, *Sarcocystis neurona* and *Cryptosporidium parvum*. The aim of this study was to investigate whether chlorothiazolides could be useful for treatment of cryptosporidiosis and a valuable alternative to NTZ. Two 5-chlorothiazolide, namely RM4850 and RM4848, featuring methyl at R4 and hydroxyl at R1 on the benzene ring, respectively, and their respective prodrugs, RM4865 and RM5038, designed and synthesized by Romark Laboratories, were shown effective against *C. parvum* development in HCT-8 cells. NTZ and three of these agents were evaluated in *C. parvum* infected immunosuppressed Mongolian gerbils. The nitrothiazolide prototype NTZ, the chlorothiazolides RM5038, RM4865 and its primary metabolite RM4850, when administered at a daily oral 400 mg/kg dose for 5 or 8 consecutive days, exhibited similar levels of inhibion of oocyst shedding. In early infected gerbils, a four-day RM5038 treatment regimen reduced oocyst shedding by 95 % when compared to control animals and appeared more effective than NTZ which exhibited a 47% oocyst shedding inhibition (P = 0.02). Chloro-thiazole derivatives, lacking nitro-group which is potentially harmful to the intestinal microbial flora, could be an alternative to NTZ, the parent compound of the thiazolide class and RM5038 is a particularly important candidate for development in humans.

Target identification of toltrazuril – review of recent results

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Toltrazuril, a symmetrical triazinone, is a very effective anti-coccidial drug registered under the original name Baycox (Bayer Animal Health GmbH, Germany) for the control of coccidiosis in poultry (Mathis et al. 2004), piglets (Mundt et al. 2007), calves (Mundt et al. 2003) and lambs (Le Sueur et al. 2009) in many countries in the world. Toltrazuril targets all intracellular stages of coccidian (Mehlhorn et al. 1984) without a negative impact on the development of natural immunity (Steinfelder et al. 2005)

Toltrazuril has been described to inhibit several enzyme-targets of the respiratory chain of mitochondria as well as two enzymes involved in pyrimidine synthesis, i.e. dihydrofolate-reductase in chicken liver and dihydroorotate-cytochrome-C-reductase in mouse liver (Harder and Haberkorn 1989). The D1 protein, which is a component of the rudimental photosystem II of apicomplexan protozoans, has been considered as a potential toltrazuril-target (Hackstein et al. 1995). Furthermore, toltrazuril has been reported to induce swelling of mitochondria and endoplasmic reticulum, as well as damages of the wall forming bodies II in macrogamonts and perturbations of the nuclear division of *Eimeria tenella* and *Neospora caninum* by light and electron microscopic studies (Mehlhorn et al. 1984; Darius et al. 2004). In a recent molecular approach (phage display), Stefan Bierbaum (Heinrich Heine University Düsseldorf 2009) screened a commercial M13 phage-based 12mer peptide library (New England Biolabs, Frankfurt M., Germany) with biotinylated toltrazuril. Toltrazuril was nitrated in a mixture of acetic acid and nitric acid and gave 2”-nitro-toltrazuril as major isomer which was purified by crystallization. Reduction of the nitrogroup by hydrogen with the aid of a palladium catalyst gave 2”-amino-toltrazuril, and subsequent reaction with thiophosgene gave 2”-isothiocyanato-toltrazuril. The isothiocyanate reacted smoothly with Biotin-PEO-LC-Amine purchased from Pierce Inc. (now part of Thermo fischer Scientific). Purity was >97% after purification by chromatography. Using the biotinylated marker a toltrazuril-binding clone could be identified which showed similarity to a putative 20.5 kDa cyclophilin-like protein in *Eimeria tenella*. Toltrazuril inhibited recombinant *Et*Cyp20.5 activity (IC50 = 1.84 µM) but did not affect activity of the cyclosporine A inhibitable *Et*Cyp 18.7 lacking the toltrazuril-binding sequence.

Cyclophilins (Cyps) are peptidyl cis/trans isomerases implicated in diverse processes such as protein folding, signal transduction, and RNA processing. BLAST and maximum likelihood analyses identified 16 different cyclophilin subfamilies within the genomes of apicomplexan parasites (Krücken 2009). Many of the putative apicomplexan cyclophilins are predicted to be nuclear proteins. The identification of Cyp subfamilies that are specific for lower eukaryotes, apicomplexa, or even the genus Eimeria is of particular interest since these subfamilies are not present in host cells and might therefore represent attractive drug targets.

Stefan Bierbaum (2009): University of Düsseldorf; Krücken et al (2009): Parasites & Vectors 2:27

Posters

Seroepidemiological study of *Toxoplasma gondii* infection in sheep and goats from the North of Portugal

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Infection with *Toxoplasma gondii* is an important cause of foetal mortality in small ruminants and the frequent origin of endemic and epidemic abortion outbreaks leading to serious economic and reproductive losses. Simultaneously, consumption of undercooked meat from sheep and even goats was identified as a major risk factor for *T. gondii* infection in humans. Between March 2008 and March 2010, blood serum was obtained from 119 sheep and 184 goats from the North of Portugal. All the sampled animals were intended for human consumption. Sera were tested for IgG at the dilutions of 1:20 (cut-off), 1:400, 1:1600 and 1:6400. A commercial kit was used (Toxo-Screen DA®, bioMérieux, Lyon, France). Antibodies to *T. gondii* were detected in 40 (33.6%) out of 119 sheep with titres of 1:20 in 16, 1:400 in one, 1:1600 in three, and 1:6400 or higher in 20 animals. Antibodies to *T. gondii* were found in 34 (18.5%) out of 184 goats, with titres of 1:20 in 25, 1:400 in one, 1:1600 in four, and 1:6400 or higher in four animals. Risk factors for infection by T. gondii in sheep in descending order of their odds ratio (OR) were: age less than 19 months (OR = 49.5, 95% CI: 3.6–673.7) and age between 7 and 18 months (OR = 5, 3, 95% CI: 1.5–19.5). After multiple logistic regression analysis, breed and husbandry system were not validated as risk factors. For goats the risk factor found was age between six and 10 years (OR = 3.8, 95% CI: 1.3–11.2). This increased risk in older animals suggests a greater probability of exposure to *T. gondii* with time, highlighting the importance of environmental contamination for the infection. Measures to prevent *T. gondii* infection should be established in small ruminant production.

Seroprevalence of *Toxoplasma gondii* infection in sheep from Romania

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Toxoplasma gondii is a zoonotic parasite belonging to Phylum *Apicomplexa* with a world-wide distribution. The prevalence of infection may vary with the animal species, being higher in small ruminants and pigs, and this might have consequences for food safety. The objective of this study was to assess the seroprevalence of antibodies to *T. gondii* in sheep from Romania. Three-thousands-eight-hundred-thirteen sera sampled from sheep (3230 ewes and 583 lambs) in all regions of Romania were tested for antibodies (IgG) to *T. gondii* by a commercial ELISA kit (Chekit Toxotest, IDEXX Laboratories, Switzerland) and by an in house ELISA, at a sera dilution of 1:400. Romania lies between latitudes 43° and 49° N, and longitudes 20° and 30° E and the climate is transitional between temperate and continental, with average annual temperature of 11°C in the south and 8°C in the north. In Romania, sheep are mainly exploited in transhumant way.

The overall prevalence of *T. gondii* infection in Romanian sheep was 56.8% (2167/3813). Antibodies to *T. gondii* were found in 61% (1970/3230) ewes and 33.8% (197/583) 1 month to one-year old lambs. The lowest prevalence was noticed in south-west (32/73; 43.8%), south-east (240/544; 44.1%) and central (333/748; 44.5%) regions of Romania with a prevalence around 45%. The highest prevalence was revealed in south (128/181; 70.7%) and western (368/502; 73.3%) regions of Romania, the prevalence being higher than 70%.

In conclusion, there is an important risk of human contamination with *T. gondii* in certain regions of Romania by consumption of row meet of sheep, mainly lamb during Easter.

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Serological investigation on *Toxoplasma gondii* infection in cattle from the North of Portugal

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The ingestion of undercooked beef is considered a risk factor for acquision of *Toxoplasma gondii* infection in humans in some countries. Studies should be undertaken to improve and update the existing information on the epidemiology and real importance of toxoplasmosis in cattle. Between March 2008 and March 2010, blood serum was obtained from 161 cows (*Bos taurus*) born and raised in the North of Portugal and were intended for human consumption. Gender, breed, age in months and husbandry system (intensive, semi-intensive or extensive) of animals were recorded. Sera were tested for IgG antibodies to *T. gondii* at the dilutions of 1:100 (cut-off), 1:400, 1:1600 and 1:6400 using a commercial modified agglutination test (MAT) (Toxo-Screen DA®, bioMérieux, Lyon, France). Age of cattle ranged between 5 and 156 months, with a median age of 8.0 months (interquartile range [IQR]: 8.0-12.0). For statistical analysis, three age groups were established: calves (less than 8 months), bullocks/heifers (8 to 12 months) and adult animals (more than 12 months). Defined breeds comprised Frisian and Portuguese autochthonous cattle breeds (Arouquesa, Maronesa and Mirandesa); crossbreed included crosses of Belgian Blue, Charolais or Limousin. Antibodies to *T. gondii* were detected in 12 out of 161 (7.5%) bovines. All seropositive animals had a MAT titre of 100. A higher cut-off titre of 100 was chosen for testing bovine sera than a cut-off of 20 or 25 for other species because of uncertainty of the efficacy of different serological tests for the diagnosis of bovine toxoplasmosis. None of the variables showed a significant *p* value in the chi-square test and therefore no risk factors were assessed. Bioassays are needed to confirm the role of beef in the epidemiology of toxoplasmosis. Measures to prevent infection should include high standards of hygiene in bovine production.

Seroprevalence of *Toxoplasma gondii* in Iberian sows

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The objective of the present study was to investigate the seroprevalence of *T. gondii* infection in Iberian sows raised under extensive and intensive management conditions. Serum samples were collected from 2,000 Iberian sows belonging to 13 farms in 2010. The mean farm size was 500 sows, and 20% of sows in each farm were sampled. Three types of management systems were included: traditional extensive outdoor (5 farms), intensive with outdoor access (4) and conventional intensive indoor (4). The presence of serum antibodies specific for *T. gondii* was evaluated by two validated commercially available tests: an indirect enzyme-linked immunosorbent assay (ELISA, ID Screen Toxoplasmosis Indirect, IDVet, France) and a direct agglutination test (DAT, Toxo-Screen DA, BioMèrieux, France). The ELISA results were expressed as the average sample to positive (SP) values, and SP values above 50% were regarded as positives. The DAT results were considered positive, at dilutions of 1:32 or higher. The prevalence data were related to the management and facilities of the farms. Serum antibodies to *T. gondii* were detected in 152 sows (7.6%) by at least one of the techniques used. The mean seroprevalence rate of toxoplasmosis in Iberian sows oscillated between 5.3% (ELISA) and 6.6% (DAT). A high level of agreement was found between the two tests (kappa value=0.7). The highest (8.4%) mean individual prevalence rate found using ELISA was found for sows raised in a traditional extensive outdoor system with the poorest facilities. Sows reared under intensive management with outdoor access showed a prevalence of 5.4%. The lowest prevalence rate (1.1%) was found in sows raised in a conventional intensive indoor system with the highest facility level. The results of this study suggest that the prevalence of *T. gondii* antibodies among Iberian sows seems to be moderate-low, though some herds have high within-herd prevalence rates. The *T. gondii* prevalence was related to the facilities and the management system of the farm.

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Seroprevalence of *Toxoplasma gondii* infection in pigs from the North of Portugal

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The ingestion of infected pork is considered a major source of human infection with *Toxoplasma gondii* in some countries. We investigated the seroprevalence of IgG antibodies to *T. gondii* and risk factors associated with the infection in pigs raised and slaughtered in the North of Portugal. Between March 2008 and March 2010, blood serum was sampled from 254 slaughtered pigs. All the sampled animals had been born and raised in northern Portugal and were intended for human consumption. Serum samples were diluted 1:20, 1:400, 1:1600 and 1:6400, and tested for antibodies to *T. gondii* using a commercial kit (Toxo-Screen DA®, bioMérieux, Lyon, France). Antibodies were found in 9.8% of the pigs (25/254), with titres of 1:20 in 16, 1:400 in 8, and 1:1600 in one animal. For statistical analysis, animals were grouped into piglets (≤ 3 months), fattening (4-9 months) and breeding pigs (≥ 10 months). The most important risk factor for infection in swine was age equal or less than 3 months, followed by age equal or higher than 10 months, and the age group of 4-9 months. The group of breeding animals presented an odds ratio (OR) five times smaller than the piglets (OR = 1.0). The OR of the fattening pigs was 10 times lower than that of the piglets. The values obtained may be the result of maternal antibodies transferred via colostrum. However, the chance of horizontal transmission of *T. gondii* cannot be excluded. Health promotion programs including good hygienic measures should be adopted in order to prevent infection with *T. gondii* among pig husbandry systems.

Molecular detection and typing of T*oxoplasma gondii* isolates from brazilian slaughtered ostriches (*Struthio camelus*)

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Toxoplasmosis is a worldwide zoonosis caused by an obligate intracellular parasite protozoan, *Toxoplasma gondii*, that affect most of production animals, as ostriches (*Struthio camelus*). This infection can be transmitted by raw or undercooked meat of production animals, presenting high importance for public health. This study was aimed to determine the prevalence of *T. gondii* in ostriches and the genotypes of this parasite detected in brain of these animals. 24 Brazilian ostrich isolates from South region of Brazil were screened by Polymerase Chain Reaction (PCR), with a 200- to 300-fold repetitive sequence, and confirmed the genotype by Restriction Fragment Length Polymorphism (RFLP) using the 12 markers: SAG1, 5'-3'SAG2, nSAG2, SAG3, BTUB, GRA6, L358, c22-8, c29-6, PK1, Apico, CS3. 18S rRNA marker was also used to differentiate *T. gondii* from other apicomplexa parasites, i.e. *Neospora caninum*, *Hammondia hammondi* and *Sarcocystis* spp. All samples were identified as *T. gondii* by 18S rRNA marker but fourteen samples presented low DNA load, resulting negative in RFLP in most markers. The other 10 samples presented three different and new genotypes (30%). 1/3 (33.3%) were typed directly from ostrich brain (TgOsBr1), and the others from bioassay. All samples were not virulent to mice during 60 days post-inoculation. All animals were bred in São Paulo State, directed for meat products. The parasite load in ostrich and mice samples (bioassay) were determined by quantitative PCR (qPCR), using SYBR Green system. The results of qPCR in typed samples ranging from ~500 to 10^3 parasites.mL^{-1}. Thus, these findings support the occurrence of *T. gondii* in ostriches from Brazil, and the genotypic variability in Brazilian isolates with new types detected.

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Seroprevalence of toxoplasmosis and neosporosis in goats from Córdoba, Argentina

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Toxoplasmosis is a world- wide distributed zoonosis that causes reproductive failure in small ruminants. It is known that *Neospora caninum* also infects goats, but its importance remains uncertain. The objective of this study was to estimate the seroprevalence of toxoplasmosis and neosporosis in goats in the northwest of the province of Cordoba, Argentina. Blood samples from 2187 Creole type goats were obtained from three districts: 1) Cruz del Eje (N=766, 26 herds), 2) Pocho (N= 739, 25 herds), 3) Minas (N= 596, 35 herds) and 3 commercial Saanen dairy farms (DF) (N=150). Sera were tested for detection of antibodies to *T. gondii* and *N. caninum* by indirect inmunoflorescence antibody test (IFAT) at a dilution of 1:100. Ninety per cent of the herds were seropositive to *T. gondii*, with a seroprevalence of 33% (729/2187 IC±1.97). No significant differences were found in seroprevalence between district 1 (38.6%) and district 2 (36.9%) and DF (41.3%). Seroprevalence of district 3 (22.4%) was significantly lower. Seroprevalence for *N. caninum* was 4.2% (91/2187 IC± 0.8). Eleven, 20 and 22 herds were seronegative in districts 1, 2 and 3, respectively. Seroprevalence in districts 2 (0.9 %) and 3 (0.8%) was significantly lower than in district 1 (9.5 %) and DF (4.7 %).This region of Argentina is traditionally dedicated to the extensive production of goats for meat, and kids are weaned and sold at 45-60 days of age. Afterwards goats are milked for 60-90 days and milk sold mainly for cheese and powder milk production. The high seroprevalence for toxoplasmosis could be related to the higher contact rate between goats with domestic and wild cats resulting from these changes in goat management. Environmental and climatic differences between districts may also affect the viability of infectious forms. Different risk factors are under study.

Seroprevalence of toxoplasmosis and neosporosis in dairy goats from Buenos Aires, Argentina

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Toxoplasma gondii and *Neospora caninum* are protozoan parasite with a facultative indirect cycle in mammals and birds as intermediate hosts and felines and canines as definitive hosts, respectively. *T.gondii* has been described as an important cause of abortions in goats when infections occur during pregnancy. Scarce data about neosporosis in dairy goats were reported. The aim of this study was to determine the seroprevalence of toxoplasmosis and neosporosis and to evaluate the distribution of antibody titers in dairy goats from the province of Buenos Aires, Argentina. Blood samples from 735 goats belonging to 17 dairy farms from Buenos Aires province were collected and tested for detection of antibodies to *T.gondii* and *N.caninum* by indirect immunoflorescence antibody test (IFAT). Up to ten seropositive sera (1:100) for toxoplasmosis were randomly selected from all the herds and were tested from 1:800 to 1:6400 Seroprevalence for toxoplasmosis was 63% (463/735) and all herds were seropositives, with a range from 19.2 to 100%. From the selected seropositive sera (163), 46% were positive ≤1:800, 21%, 1:1600 and 33% ≥ 1:3200. The seroprevalence for neosporosis was 9.7% (71/735) and 14 herds were seropositive with a range from 6 to 22%. Results confirm that *T.gondii* infection is common in dairy goats from the province of Buenos Aires. Previous reports from our Laboratory and the field, suggest a high risk of abortion in four of the dairy herds studied with high titers. High seroprevalence, derived from exposition or vertical transmission, may determine a significant economic impact because of the reproductive failures and conditions for manufacture of goat milk and derivates. Although in previous studies, antibodies to *N.caninum* were detected in an aborted fetus from 1 herd of this study, more investigations should be conducted in order to know the relevance of neosporosis in dairy goats.

Neosporosis in farming fallow deer (*Dama dama*) in Poland

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Extensive serological surveys have been performed in wild species showing that *Neospora caninum* can infect a wide range of species worldwide and detection of specific antibodies is a good indicator of exposure of animals to this parasite.

Presented studies were done in the breeding station in Kosewo Górne, in the Mazurian Lake District, north-east Poland (latitude $53^0$41' North, longitude $21^0$25' East).

In this study conducted from 2008 to 2011, sera from 495 farmed fallow deer (*Dama dama*) were used. All samples were analyzed for the presence of antibodies to *N. caninum* using an enzyme-linked immunoassay (ELISA), according to the manufacturer's instruction (IDEXX Laboratories Inc., Westbrook, ME, USA) and own modifications. Negative and positive sera of red deer were kindly provided by Dr. J.P. Dubey, USDA, Beltsville, MD, USA. SDS-polyacrylamide gel electrophoresis and Western blot analysis were performed according to Björkman et al. (1994) and Cabaj et al. (2005) using Bio-Rad System.

Antibodies to *Neospora* antigens were detected in 42 examined sera (6.66%). However detected seroprevalence varied, and achieved 3.02%, 15.3% and 9.5% in 2008-2009, 2010 and 2011, respectively. In these sera Western blot analysis revealed seroreactivity against immunodominant *N. caninum* antigens 37, 25 and 16 kDa, however in some sera additional stained bands were visible. To avoid the false positive results or cross-reactions, all sera exceeding 0.2 absorbance in ELISA test were verified in Western blot using *T. gondii* antigen. None of examined sera gave positive bands characteristic for *T. gondii* antigen.

Basing on our earlier experience (Cabaj et al., 2005), on *in vitro* isolation of the first *N. caninum* isolate from European bison (*Bison bonasus bonasus* L.) we have isolated two new isolates from the peripheral white blood cells from positive fallow deer. The further studies are in progress.

First data regarding the seroprevalence of *Neospora* spp. Infection in horses from Transylvania, Romania

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The two species from *Neospora* genus, *Neospora caninum* and *Neospora hughesi* have been mentioned as a cause of infection in horses (Lindsay, 2001). Even if neosporosis is considered a very important parasitic disease in cattle and dogs worldwide scarce aspects are available regarding the pathogenity and transplacentary infection with parasites of this genus (Dubey et al., 1999; Pitel et al., 2003). Still remains to be cleared the abortive role of species from *Neospora* genus in pregnant mares (Duarte et al., 2004). This is the first study in Romania performed for detection of anti-*Neospora* spp. antibodies in sera samples collected from horses. 82 horses from Transylvania, Romania were taken under study. At the moment of their slaughtering blood samples were collected and analyzed using an *in house* IFAT method. The results were assessed at the cut-off dilution of 1:50 and for positive samples consecutive dilutions were made until final dilution of 1:200. 32.2% of the animals were positive to *Neospora* spp. infection. The highest prevalence was obtained for horses with ages between 1 and 5 years old (30.4%). Anti-*Neospora* spp. antibodies in horses have been reported in different location of the world: USA and New Zeeland (Dubey, 2003; Cheadle et al., 1999; Vardeleon et al., 2001), South Korea (Gupta et al., 2002), France (Pitel et al., 2003), Italy (Ciaramella et al., 2004) and Brasil (Hoane et al., 2006). It has been noticed that their prevalence varies considerably according on the geographical area, age categories and studies made on the same region.

Bovine besnoitiosis in the Alentejo region

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Bovine besnoitiosis is a protozoal disease of cattle caused by *Besnoitia besnoiti*, an intracellular obligatory parasite belonging to the cyst forming coccidia. The infection with *B. besnoiti* can cause severe illness both during the acute and chronic phases, which may lead to considerable economic losses. Bovine besnoitiosis was recently considered an emergent disease in Europe, due to the increasing prevalence and geographical expansion in several European countries. In Portugal, scattered epidemiological data link the disease to the Alentejo region, but prevalence and geographic distribution of the disease in this region remain unknown. The present study arose from the need to further evaluate the extent of besnoitiosis in the bovine population from Alentejo and consolidate existing epidemiological information. With this purpose, a serological survey was carried out in 2012 involving 21 cattle farms, randomly selected from 11 counties in Alentejo. Serum samples were obtained under the scope of the national Eradication Program of Bovine Brucellosis. A total of 3583 animals, corresponding to 0.9% of the bovine population in this region, were screened for anti-*B.besnoiti* specific antibodies by the modified agglutination test (B-MAT). Positive sera by B-MAT (159) were confirmed by indirect immunofluorescent antibody test and 104 were positive. This serological survey showed a test prevalence of 2.90% (true prevalence 95%CI: 3.24% - 3.26%, considering overall diagnostic SE=0,8933 and SP=1,0). Seven out of 21 farms (33.3%) were positive, belonging to 6 counties (54.5%). Within herds prevalence was 0.6%, 0.8%, 0.9%, 1.8%, 4.8%, 25.0% and 54.4%. The present data, combined with previous information on case reports and serological studies, clearly show a wide distribution of *B. besnoiti* in the Alentejo region.

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Serological survey of *Besnoitia* spp. infection in Spanish wild ruminants

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Besnoitia besnoiti and *Besnoitia tarandi* have been reported to affect domestic and wild bovids (cattle, wildebeest and impala) and cervids (caribou and reindeer), respectively, causing similar characteristic clinical signs and lesions. Both *Besnoitia* species have been reported in different countries, but the link between the sylvatic and domestic life cycles of *Besnoitia* spp. in wild ruminants and cattle remain unknown. The aim of the present study was to evaluate the presence of specific antibodies to *Besnoitia* spp. in Spanish wild ruminants. A wide panel of sera from red deer (*Cervus elaphus*) (n=732), roe deer (*Capreolus capreolus*) (n=124), chamois (*Rupicapra rupicapra*) (n=170) and mouflon (*Ovis musimon*) (n=19) collected from different locations in northern, central and southern mainland Spain was analysed. Beef cattle are present in all sampled areas, and bovine besnoitiosis has been widely reported in some of these areas (e.g., Pyrenees and central Spain). Sera from red deer and roe deer were first examined by an Enzyme-Linked Immunosorbent Assay (ELISA) standardised with positive and negative control sera from several Cervidae species (100% Se and 96.5 % Sp). Chamois sera were tested using a previously reported ELISA validated for bovine sera (97 % Se and 95% Sp). Mouflon sera were evaluated using the previously mentioned ELISA with protein G as the conjugate. Positive ELISA results were confirmed a posteriori using a tachyzoite-based western blot. Twenty serum samples from red deer and nine roe deer were seropositive by ELISA, and all samples from chamois and mouflon were seronegative. *B. besnoiti* infection was clearly confirmed by western blot in only one red deer and one roe deer from the Spanish Pyrenees, where the disease is traditionally endemic in cattle.
This is the first serological report of *Besnoitia* spp. infection in Spanish wild ruminants, and the results show that the infection is present at least in red deer and roe deer. Thus, wild ruminants from regions endemic for bovine besnoitiosis should be further investigated in depth because these animals may be putative reservoirs of the parasite.

Novel *Besnoitia* in Australian macropods

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Cases of epistaxis in sub adult Western Grey kangaroos (*Macropus fuliginosus*), Red kangaroos *(Macropus rufus)* and Common Wallaroo *(Macropus robustus)* have been reported recently by wildlife carers in South Australia. Some clinical cases were reported to have died from severe epistaxis but no necropsies were performed.

Besnoitia-like parasites were visualised microscopically in Diff Quik stained smears made from nasal swabs and flushes obtained from individuals (3) examined in the autumn of 2011 and 2012. These contained small numbers of hypertrophied host epithelial cells, each with an enlarged displaced nucleus and a large (> 100 um) intracytoplasmic parasitophorous vacuole enclosing closely packed small (~4um long) protozoal zoites. These features resemble *Besnoitia* spp. Organisms were not observed in nasal swabs examined in wintertime.

In the absence of positive species identification of the parasites, molecular analyses and serological testing is being conducted on stored samples from affected animals and on further animals in the same population of macropods to ascertain the identity and extent (prevalence) of the infection with this parasite.

In Australia, besnoitiosis has never been reported from cattle, but serological assays of cattle conducted in South Australia have recently demonstrated some cross-reactions in a commercially available enzyme-linked immune-sorbent assay for *B. besnoiti* (Nasir et al., 2012). Since besnoitiosis has emerged as a disease of cattle in Europe (Cortes et al., 2006) and spread to Northern European countries such as Germany, France and Italy (Gollnick et al., 2010; Lienard et al., 2011; Rostaher et al., 2010) the study of *Besnoitia* in kangaroos is important in preparation for its emergence in other animal species.

Cortes et al. (2006) Vet Parasitol 141, 226-233;
Gollnick et al. (2010) Vet Rec 166, 599;
Lienard et al. (2011) Vet Parasitol 177, 20-27;
Nasir et al. (2012) Veterinary parasitology 186, 480-485;
Rostaher et al. (2010) Vet Dermatol 21, 329-334.

Cryptosporidium infection in beef calves in Sweden

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The protozoan parasites *Cryptosporidium* spp are clinically important pathogens causing gastrointestinal disease and diarrhea in a variety of species including humans and cattle. One of the *Cryptosporidium* species infecting cattle, *C. parvum*, is zoonotic and can be transmitted between cattle and man. This species is primarily found in preweaned calves. The role of grazing cattle as vectors and disseminators of infection, and to which extent they may contribute to waterborne *C. parvum* infection in humans is a topic for debate. In Sweden, only beef calves and not calves in dairy herds are kept on pasture.

The aims of this study were to investigate how common *Cryptosporidium* infection is in Swedish beef cattle herds and to explore which species and subtypes that occur.

Twenty four beef cattle herds were enrolled in the study. Each herd was visited once from April to June 2012. Faecal samples were collected from all calves younger than 3 months, and information about the herd and management routines were registered. The samples were cleaned and concentrated by saline-glucose flotation, stained with monoclonal anti-*Cryptosporidium* antibodies and analysed for presence of oocysts by epifluorescence microscopy. The lower detection limit of this method is 400 oocysts per gram faeces (OPG). Molecular analysis of the ssrRNA and GP60 loci will be performed.

A total of 276 calves from 1-90 days of age were sampled and *Cryptosporidium* oocysts were detected in 98 of them (36 %). Positive calves were identified in 23 (96 %) herds. Oocyst counts in positive samples were 400 to 2.6×10^6 OPG. Molecular and epidemiological data will be presented and discussed at the conference.

Sarcocistiosis of pigs, cattle, sheep and dogs in the some regions of Serbia

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Sarcocystis spp. in meat slaughtered animals (catlle, sheep, pigs), by directs methods of diagnosis was investigated and the prevalence of sarcocystiosis in dogs was examined in six regions in Serbia.

Samples of muscular oesophagus, diaphragma, tongue and heart was taken from line of the slaughter in the period of the five years. Sarcocystiosis was diagnosed by the method of compression and histological analysis by staining muscle sections with H & E. The number of microcysts per gram of the muscle in pigs and sheep, was determined by stereometrical analyses and the microcysts' volume was measured according to the formula for a rotatory elipsoid conus. Oocysts in dog faeces were determined by ZnCL2- NaCl flotacion.

Sarcocysts are located as macro and micro cysts in the sheep and more ofen (40.1% till 99,5%) than to cows (21% to 54.1%). Sarcocysts by intensity are found predominantly in oesophagus but at least in diaphragma, heart and tongue slaughtered animals. The comparison of the porcine infection with that in sheep showed a significant difference in the number of microcysts per gram of the cardiac muscle and diaphragm ($P<0.01$), and no significant difference in the tongue muscles ($P>0.05$). The comparative of microcysts in sheep and pigs showed a very significant difference in the volume of microcysts recovered from the cardiac muscles ($P<0.001$), being insignificant from the tongue and diaphragm muscles of sheep and pigs ($P>0.05$).

Sarcocystiosis was established in 15.9% (63/395) market-weight pigs from large state-owned farms, and in 33.1% (130/393) from private small pig farms ($P>0.0001$). In sheep, *Sarcocysis* infection was confirmed in 34.4% (31/90) lambs, and in even 97% (223/230) adult sheep ($P>0.0001$). An age-dependant increase in infection rates ($P>0.0001$) was observed in cattle as well, ranging from a prevalence of 4.8% (4/83) in calves, 54.7% (75/137) in "baby beef" cattle, and 37.8% (106/280) in adult cattle.The prevalence of sarcocystiosis in dogs was 2.5% (11/448), with no significant differences among dogs from differen settings.

Species of the *Eimeria* parasites lambs from Brazil

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The aim of this study was to investigate the occurrence of infection, correlating with age and to identify which species of *Eimeria* parasitize sheep from the City of Alambari, Brazil. We collected 142 fecal samples from lambs up to one year of age, males and females, and various races. According to the age, the animals were divided into Group 1 (n = 33) 1-3 months, group 2 (n = 20) 4-7 months and group 3 (n = 89) 8-12 months. Faecal samples were quantitatively analyzed by the method of oocyst count per gram of feces (OOPG). Aliquots were stored in a positive conical Falcon tube containing solution of potassium dichromate and 2.5% for seven days at room temperature until sporulation for subsequent identification of species of said coccídeo. Proceeded to the different species according to shape, color, presence or absence of micropyle and micropylar cap, and the size of the oocysts, as measured using an optical microscope designed the ocular micrometer. Of the total 32 samples tested were positive, in which, we identified the following species: *E .crandallis* (9.4%); *E. faurei* (6.3%); *E. marsica* (28.1%); *E. ovinoidalis* (56.3%); *E. pallida* (25%); *E. parva* (12.5%) and *E. weybridgensis* (75%), observing the presence of co-infection in all samples. The species found most often were *E. weybridgensis* and *E. ovinoidalis*. In terms of age group 1 was observed occurrence of 37.5% (12/32); 40.6% (13/32) in group 2; 21.9% (07/32) in group 3, showing that the said coccídeo was present in all age groups therefore being statistically significant p <0.0001. It is concluded that infection was seen in animals and group 2 was the most affected. So there is need to implement a program of parasite control in this region, since coccidiosis in lambs may therefore lead to economic losses.

Eimeria excretion in goats: peri-parturients and kids

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Goat milk and meat have higher market value than sheep products; nevertheless goat population (half million animals) represents about 20% of sheep population. Coccidiosis represents a major health and production problem in goats: body weight losses, cost of treatments and death in kids. On the present work the *Eimeria* excretion and its health implications in the kids were followed up. Two goat farms in south Portugal were followed up since one week before until 3 months after kidding, in a weekly basis: feces samples, blood samples and body weight measurements. From each farm 7 kids and 6 dairy goats (progenitors) were monitored for 12 weeks and 5 weeks. *Eimeria arloingi* and *E. alijevi* were the most prevalent species in both groups. *E. ninakohlyakimovae* was as well present in the dairy group and *E. caprovina* in the kids group. Dairy goats revealed a decrease in OPG numbers in consecutive weeks after parturition, but the differences were not considerable. Significant differences were found between the two farms; the herd with less insolation and higher humidity presented higher OPG counts in the two groups ($P < 0.05$). Kids started shedding 2 and 3 weeks after birth and an increase in total OPG was observed every 2 or 4 weeks (in peaks), with numbers reaching 340.000 OPG. *E. arloingi* presented high values every 3 or 4 weeks. Other species presented a similar pattern in the two farms: *E. christenseni*, *E. alijevi* and *E. caprovina*. The Concomitant infections were detected in kids since the third week of age in both farms, identifying 2 to 7 *Eimeria* species per sample. As kids showed to be the most important source of environmental contamination, kids born later on the kidding season, may even present higher risk of coccidiosis if no appropriate health management is carried on.

Distribution and economic impact of coccidiosis on small scale commercial farms in Africa

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Coccidiosis, a host species-specific intestinal disease caused by *Eimeria* parasites, causes substantial production losses in the poultry industry worldwide. Nonetheless, the impact of coccidiosis on small-scale poultry production and its influence on poverty in Sub-Saharan Africa remains unclear. Our objective was to examine the distribution and economic impact of *Eimeria* within small-scale commercial poultry farms in Africa.

Faecal samples and data on production parameters and farm management were collected from small-scale (<2000 birds) commercial broiler and layer farms within Ghana, Tanzania and Zambia. The gross margin and enterprise budget bird^{-1} year^{-1} were calculated to assess individual farm profitability. *Eimeria* species distribution was determined through faecal microscopy, confirmed by *Eimeria* species-specific PCR. Field samples were screened for the presence or absence of each of the seven *Eimeria* species that cause coccidiosis in the chicken, in addition to the three cryptic strains identified from surveys of Australian poultry as 'operational taxonomic units (OTUs) X-Z'.

Eimeria were found to be widespread within African poultry and were present on 75% (60/80) farms sampled. Species complexity was comparable to that of Europe with all seven *Eimeria* species detected and 35% (28/80) farms concurrently infected by multiple species. Intriguingly, sequences consistent with the presence of OTUs X and Z were identified for the first time outside of Australia. Farmers reported awareness of clinical coccidiosis and mortality rates of up to 80%. The profitability of farms varied substantially by country and production type, with gross margins ranging from -21.88 to 52.30 USD bird^{-1} year^{-1} in layer systems and from -4.01 to 8.01 USD bird^{-1} year^{-1} year in broiler systems. Relative pathogenicity of species present was associated with farm profitability ($p<0.01$, Chi-square).

Further studies are required to characterise the *Eimeria* population within Africa, their economic impact on poultry farms and to identify cost effective control strategies and interventions.

Drug-resistance to anticoccidials of *Eimeria* spp. field isolates collected in 2010 from broiler chickens farms in Romania

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Twelve field isolates of *Eimeria* spp. sampled from six broiler chickens farms (2 isolates/farm) in Romania were subject of anticoccidial sensitivity test in a battery trial. The *Eimeria* spp. samples were isolated from faeces of 25-32-days-old chickens and screened against anticoccidial drugs used in the farms of origin, as follows: lasalocid (125 ppm) in farms A and D; narasin/nicarbazin (50 ppm) in farms A and B; monensin (125 ppm) in farm E; salinomycin (60 ppm) in farm C and maduramicin (5 ppm) in farm F.
For each *Eimeria* spp. isolate was designed experimental groups of 10 chickens in duplicate: uninfected untreated control (UUC) group; infected untreated control (IUC) group and infected treated (IT) group. The experimental infection was made when chickens were 14-days-old with $1x10^5$ *E. acervulina* oocysts and with $1x10^4$ *E. tenella* oocysts. The medicated feeds was gave to treated groups with 2 days prior to infection till the end of the study. Lesion score was performed at 7 days post-inoculation in all chickens. The anticoccidial-sensitivity profile was based on the reduction percentage of the mean lesion score of the IT group compared with the IUC group as follows: 0 to 30% coccidial resistance; 31 to 49% partial resistance, and 50% or more full sensitivity.
All *Eimeria* isolates contained *E. acervulina* and *E. tenella.* In four farms (A, C, D, E) out of six was noticed drug-resistance to used anticoccidials. In farms with drug-resistance only in one farm (A) the drug-resistance was present in both houses and for both *Eimeria* species. *E. acervulina* showed resistance in 7 (farms A, D and E) out of 16 situations and partial resistance in 3 (farms A and D) cases. *E. tenella* showed resistance in 7 (farms A, C and E) out of 16 situations and partial resistance in one cases (farm E).

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The infection dynamics of *C. bovis* in a Swedish dairy herd

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Cryptosporidium spp are intracellular parasites in different species, including humans and cattle. *C. parvum* is considered one of the most common causes of calf diarrhea and is also a clinically important pathogen in humans. It has been shown that what has previously been known as *C. parvum* in cattle are indeed three different, species; *C. parvum, C. bovis* and *C. ryanae. C. parvum* is the species considered to be pathogenic in cattle whereas the others are thought to cause subclinical infection. In Sweden, *C. bovis* is the dominating species in young calves, and we find high numbers of *C. bovis* oocysts but no *C. parvum* or other diarrheal agents in many samples from diarrheic calves. As part of a PhD project, the *Cryptosporidium* species distribution pattern in calves from birth to calving in a dairy herd is investigated. The farm is an organic dairy farm with 120 milking cows in a loose housing system. 15 heifer calves were enrolled in the study during their first week of life. They will be examined weekly to record body condition score, bedding, illness etc. until two months of age and thereafter once a month until they calve or are brought to slaughter, and fecal samples will be collected on every visit.

The fecal samples are cleaned and concentrated by saline-glucose flotation, stained with monoclonal anti-Cryptosporidium antibodies and analysed for presence of oocysts by epifluorescence microscopy. The lower detection limit of this method is 400 oocysts per gram feces (OPG). Molecular analyses of the ssrRNA and GP60 loci will be performed.

This project is a work in progress and from February to July 2012, a total of 250 samples have been collected from the calves and molecular analyses have been performed on 24 of these and the rest are underway.

Infection by *Cryptosporidium* spp. in lambs

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The aim of this study was to verify the occurrence of infection by *Cryptosporidium* in stool samples from lambs up to one year old in the city of Alambari, Brazil. The samples were examined by Kinyoun technique and PCR (polymerase chain reaction). Fecal samples from 211 sheep from all the farms, males and females of various breeds were collected and divided into two aliquots, the first for the manufacture of films for analysis by the modified Kinyoun method and another aliquot was frozen "in nature "-20°C until running the PCR. The slides stained by the Kinyoun technique were read by optical microscopy magnification of 400X and 1000X for detection of *Cryptosporidium* spp.. All samples were sent for DNA extraction from oocysts using the "QIAmp DNA stool mini kit" (Qagen ®), according to the manufacturer's protocol. To the reaction nested-PCR amplified fragment of the 18S subunit ribosomal RNA gene of *Cryptosporidium* primers used were 5´ TTC TAG AGC TAA TAC ATG CG 3' and 5' CCC ATT TCC TTC GAA ACA GGA 3'to the primary reaction (1325 bp) and 5' GGA AGG GTT GTA TTT ATT AGA TAA AG 3' and 5´ AAG GAG TAA GGA ACA ACC TCC A 3´ the secondary reaction (bp 826-840) and the reaction according to the protocol Xiao et al. (2000). For the Kinyoun technique was not observed in animals oocysts as PCR was 15.2% (32/211) of positive results in the amplification of *Cryptosporidium* DNA. In conclusion, *Cryptosporidium* infection was detected in sheep in the city of Alambari, which represents a potential risk of environmental contamination. Studies on the molecular characterization are needed to understand the epidemiology and zoonotic potential of these isolates.

Slacked lime – a disinfectant against *Cryptosporidium* sp.?

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Cryptosporidium sp. are single cell parasites causing diarrhoea in different animals, including man and cattle. *Cryptosporidium* infections are prevalent in Swedish dairy herds and are together with rota-virus the most common cause of diarrhoea in calves. Diseases in calves are a significant animal health problem with economic consequences for the owner. It is therefore of great importance for the animal owner to be able to evade risk of infection. *Cryptosporidium* oocysts are resistant to commonly used chlorine based disinfectants and the use of slacked lime against *Cryptosporidium sp.* would be of great interest for animal owners, since it is not toxic to handle, as compared to many other disinfectants, and also have a drying effect on the surface. We have in this study evaluated how efficient slacked lime is on disturbing viability and survival of *Cryptosporidium* oocysts, the infective stage of the parasites life cycle. We investigate the optimal concentration and time of exposure for an efficient deactivating effect of the oocysts. Isolated *Cryptosporidium parvum* from Moredun isolate were exposed to different concentrations of slacked lime (0.0, 0.1, 0.25 and 0.5 kg/m^2) during different time intervals (1, 4, 16 and 48 h.). Preliminary results suggest an effect of slacked lime on inactivation of *C. parvum* oocysts. We see alterations in shape, infection capacity and survival of the oocysts. Updated results from this study and recommendations on slacked lime as a disinfectant against *Cryptosporidium* on farm level will be presented.

Overview on control options for tropical theileriosis in Portugal

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Tropical theileriosis is a tick-borne hemoprotozoan disease that causes important health problems in cattle. The etiological agent is the apicomplexan parasite *Theileria annulata* that occurs in southern Europe, North Africa and certain parts of southern Asia. The disease, as well as the carrier state, cause significant production losses, due to mortality, treatment costs, decreased average daily weight gain and milk production. In Portugal, with a cattle population of approximately 1.3 million animals, the authors' epidemiological study showed a prevalence of 21.3% *T. annulata* infected animals that ranged from 3.3% in the North and 33.5% in Lisbon and Tagus Valley.
In endemic areas like Portugal, tick vector control plays an important role in reducing the impact of theileriosis. In our country, *Hyalomma marginatum* and *H. lusitanicum* ticks are supposed to be the main vectors. The regular use of acaricide on animals and housing, especially during peak tick activity season should help disease control. Nevertheless acaricides may contaminate milk and meat and may be a risk for human health. In several countries this strategy is likely to be more effective when used in an integrated approach with attenuated cell-line vaccines. Vaccination is an efficient measure leading to a decrease in disease incidence but is not used in Portugal. Recently, research groups are considering pre-existing genetic resistance or tolerance of certain cattle breeds as promising since conventional strategies are failing to control the disease.
Farmers and veterinary practitioners should also be aware of the existence of a high prevalence of carrier animals infected with *T. annulata* in our country, given the potential threat this pathogenic parasite represents to the Portuguese livestock industry. In this work we reviewed the control strategies since there is no available effective treatment for this disease in Portugal.

The core mouse response to infection by *Neospora caninum* as defined by gene set analyses

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Neospora caninum is recognised as an important cause of abortion and fetal death in cattle and it is believed that the development of vaccines against this parasite will prosper with the creation of new knowledge on host responses to infection. However the role of the host response as a contributor to fetal loss has yet to be investigated. In this study the BALB/c and Qs mouse responses to infection by *N. caninum* was investigated by gene set (enrichment) analyses of microarray data. A variety of approaches were used including GSEA, MANOVA, Romer, subGSE and SAM-GS to study the contrasts *Neospora* type, Mouse type (BALB/c and Qs) and time post infection (6 hours post infection and 10 days post infection). The analyses show that the major signal in the core mouse response to infection is from time post infection and can be defined by gene ontology terms Protein Kinase Activity, Cell Proliferation and Transcription Initiation. Several terms linked to signaling, morphogenesis, response and fat metabolism were also identified. At 10 days post infection genes associated with fatty acid metabolism were identified as up regulated in expression. The value of gene set (enrichment) analyses for the analysis of microarray data is discussed.

Integration of reporter genes (YFP or Lac-Z) in pyrimethamine or chloramphenicol resistant *Neospora caninum*

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Neospora caninum is an Apicomplexan parasite directly related to abortion and fertility losses in cattle. Genetic manipulation is very well developed for *Toxoplasma gondii* and *Plasmodium* spp, offering several tools for studies involving invasion and replication processes. Our laboratory is working on developing genetic options for *N. caninum,* therefore we have recently developed two forms of stable insertion of genes by resistance against chloramphenicol or pyrimethamine. For the resistance against pyrimethamine, the coding sequence of NcDHFR-TS (Dihydrofolate reductase- Thymidylate synthetase) was point mutated in two aminoacids, serine 36 to arginine (M2) and tyrosine 83 to aspartic acid (M3) generating DHFRM2M3. The DHFRM2M3 flanked by the promoter and 3' UTR regions of Ncdhfr (Ncdhfr-DHFRM2M3) conferred resistance against pyrimethamine after transfection. The chloramphenicol resistance was obtained after the transfection of tachyzoites with the chloramphenicol acetyltransferase gene (CAT) flanked by the promoter and 3' UTR region of Ncdhfr (Ncdhfr-CAT). The cassettes Ncdhfr-DHFRM2M3 and NcDhfr-CAT were ligated to the reporter genes Lac-Z (β-galactosidase enzyme) or YFP (yellow fluorescent protein) controlled by the *N. caninum* tubulin promoter (NcTub-tetO/Lac-Z or NcTub-TetR/YFP) and was transfected in *N. caninum*. The tachyzoites transfected with Ncdhfr-DHFRM2M3/NcTub-tetO/Lac-Z or Ncdhfr-CAT/NcTub-tetO/Lac-Z and selected with, respectively, pyrimethamine or chloramphenicol, expressed Lac-Z and allowed the detection and quantification of tachyzoites with the CPRG reaction or visualization after X-gal precipitation. The cassettes Ncdhfr-DHFRM2M3/NcTub-TetR/YFP or Ncdhfr-CAT/NcTub-TetR/YFP (YFP expression) after transfection and selection with pyrimethamine or chloramphenicol resulted in fluorescent tachyzoites, visualized by confocal microscopy. The stable integration of reporter genes in *N. caninum* is the first step for gene control and functional studies in this parasite, methodologies commonly applied in *T. gondii* and *Plasmodium* spp, but very underdeveloped in *N. caninum.*

The genome of the mouse parasite *Eimeria falciformi*

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We sequenced the genome of *Eimeria falciformis* as part of a programme to establishing this parasite of mice as a model for other species of the genus *Eimeria,* causing disease in poultry as well as for other coccidian parasites of veterinary and medical importance. We demonstrate the high quality of our genome assembly in comparison to data available for other *Eimeria* species and analyse the virtually complete genome sequence of *Eimeria falciformis* in comparison to genome sequences throughout the Apicomplexa. We investigate base composition features of non-coding sequence and of protein-coding genes and map the emergence of genomic features to a phylogenetic tree. Clustering of proteins into homologous families and phylogenetic stratification of these combined with functional annotation allowed the identification of putative novel, restricted and lost processes in the genus *Eimeria* and in the class Coccidia. We will discuss in how far - from the perspective of the genome - similarities, novelty and loss allow the use of Eimeria falciformis as a model for other apicomplexan parasites.

Differential gene expression in extra- and intracellular life cycle stages of the mouse parasite *Eimeria falciformis*

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Coccidiosis caused by parasites of the genus *Eimeria* is considered as one of the economically most important diseases in the poultry industry. Due to the development of resistances against available anticoccidials a better knowledge of parasite biology and host immune responses is needed to design new control strategies. Additionally, in contrast to other apicomplexans, Eimeria parasites do not change between hosts, making them an attractive model system to study molecular mechanisms of apicomplexan biology throughout the whole parasite life cycle, including both asexual and sexual stages.
To obtain a dynamic picture of gene expression, we performed sequential high throughput transcriptome analysis of RNA isolated from extracellular parasite stages (sporozoites and unsporulated oocysts) and intracellular stages of the mouse parasite *E. falciformis* at different time points (3, 5 and 7 days post infection). Our analyses reveal dramatic changes of gene expression during the course of infection and provide insights into the biology of Eimeria parasites with a hitherto unprecedented precision.

Stage specific reporter gene assays in *Eimeria nieschulzi* and their control

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One of the keys to understand the biology of coccidia is their ability to survive adverse environments and conditions. Surrounded by two oocyst walls and protected by sporocysts, eimerian parasites are masters in overcoming mechanical and chemical stress. The challenge of investigating oocyst wall formation by molecular methods is the stage specify of this process.
In this study, the *Toxoplasma gondii* DHFR-TSm2m3 pyrimethamine resistance gene was fused with the yellow fluorescent protein (YFP) encoding sequence to provide continuous pyrimethamine resistance and fluorescence in the *Eimeria* parasite from a single transcript. The permanent YFP signal of transgenic parasites allows differentiating transgenic parasites from wild type parasites throughout the entire life cycle. Within three passages under pyrimethamine treatment, a strain with 100% transformed sporulated oocysts of the parasite was isolated. This new method provides the potential to produce and monitor transgenic *Eimeria* strains without additional fluorescence activated cell sorting (FACS). The chimeric fluorescent reporter is utilized as a continuous internal control for plasmids containing stage specific promoter and genes. An *Eimeria tenella* gamogony gene specific regulatory sequence was utilized to confer macrogamont specific tandem dimer tomato (tdtomato) reporter gene expression in *Eimeria nieschulzi*.
This chimeric reporter system was applied to investigate wall formation and it's involved proteins in more detail.

Towards an *in silico* vaccine discovery pipeline for Apicomplexa of farm animals

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An *in silico* vaccine discovery pipeline based on reverse vaccinology encompasses a series of steps that exploits the genomics, transcriptomics, and proteomics of a pathogen. The desired output from such a pipeline is a list of proteins that are deemed to represent vaccine candidates based on rational criteria such as antigenicity and cellular location of the proteins. There are several successful applications of reverse vaccinology to the discovery of subunit vaccines against prokaryotic but not eukaryotic pathogens. In this paper a framework for an *in silico* pipeline is discussed as a guide to high-throughput vaccine candidate discovery for eukaryotic pathogens, such as the Apicomplexa of farm animals. The pipeline is based on the principle of reverse vaccinology and is constructed from freely available bioinformatics programs. The overriding aim of the pipeline is to generate through computational processes of elimination and evidence gathering a ranked list of proteins based on a scoring system. These proteins may be either surface components of the target pathogen or are secreted by the pathogen *and* are of a type known to be antigenic. No perfect predictive method is yet available so the highest scoring proteins from the list require laboratory validation.

The epidemiology of *Cryptosporidium* species and genotypes in Scottish cattle populations

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In the North East of Scotland the beef industry experienced significant increases in disease and calf mortality attributed to *Cryptosporidium* infection during 2009 and 2010, where one farmer reported that he lost 30% of his calves due to *Cryptosporidium* infection.

The studies presented here aim to address if severity of disease can be attributed to specific *Cryptosporidium* species and genotypes. In order to investigate this, a nested multiplex species specific PCR (nmss-PCR) was developed that allows the distinction of the most commonly found *Cryptosporidium* species found in cattle: *C. parvum, C. bovis, C. ryanae* and *C. andersoni*. Samples that tested positive for *C. parvum* were genotyped, using the gp-60 locus using a sequencing approach.

These molecular tools were applied to faecal samples, obtained from 39 farm beef farms in the North East of Scotland. The results show that 80% of farms had *Cryptosporidium*, that the most frequently identified species in calves was *C. parvum* and that adult cattle only rarely shed detectable levels of *C. parvum*. Analysis of the gp-60 genotype, of the *C. parvum* positive samples, revealed that IIaA15G2R1 was the most common genotype, while one farm that suffered from more severe disease had an unusual genotype.

A longitudinal study of 30 neonatal calves from a dairy farm, with a history of *C. parvum* infection both in cattle and visiting students, has shown that calves shed oocysts of different *Cryptosporidium* species in an age dependent manner. Calves less than 4 weeks of age tended to shed only *C. parvum* oocysts. After 4 weeks *C. bovis* was also detected, sometimes in mixed infections. At 3 month, the same animals shed mostly *C. bovis* and *C. ryanae* oocysts but surprising when the same animals were re-sampled at 6 month, the majority shed *C. parvum* oocysts again. Samples from adult cattle from the same farm revealed that this farm is also infected with *C. andersoni.*

Molecular characterization of *Cryptosporidium* spp. in fecal samples of lambs in the south of the state of São Paulo, Brazil

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The aim of this study was to obtain epidemiological information through the molecular characterization of *Cryptosporidium* spp. in fecal samples from lambs up to one year of age in South state of Sao Paulo, Brazil. The samples were evaluated by nested PCR technique (polymerase chain reaction). Fecal samples from 193 sheep from four farms, males and females of various breeds were collected, and an aliquot was frozen "in nature" -20°C until running the PCR. All samples were sent for DNA extraction from oocysts using the "QIAmp DNA stool mini kit" (Qagen®), according to the manufacturer's protocol. To the reaction nested-PCR amplified fragment of the 18S subunit ribosomal RNA gene of *Cryptosporidium* primers used were 5´ TTC TAG AGC TAA TAC ATG CG 3' and 5' CCC ATT TCC TTC GAA ACA GGA 3'to the primary reaction (1325 bp) and 5' GGA AGG GTT GTA TTT ATT AGA TAA AG 3' and 5´ AAG GAG TAA GGA ACA ACC TCC A 3´ the secondary reaction (bp 826-840) and the reaction according to the protocol Xiao et al. (2000). PCR technique was 9.85% (19/193) of positive results in the amplification of *Cryptosporidium* DNA. The genotypic analyzes revealed *Cryptosporidium xiaoi* in fifteen samples; *Cryptosporidium ubiquituim* in three and *Cryptosporidium meleagridis* in only one sample. In conclusion, *Cryptosporidium* infection was detected in sheep in the south state of Sao Paulo, which represents a potential risk of environmental contamination.

Molecular characterization of *Cryptosporidium* spp. in buffalo calves from Brazil

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The aim of this study was to determine the occurrence and do the molecular characterization infection by *Cryptosporidium* spp. in buffalo calves in the state of São Paulo, Brazil, were collected 222 fecal samples from animals Murrah, with up to six months old. The samples were evaluated by the technique of Kinyoun and by nested-PCR reaction for the amplification of fragments of DNA subunit 18S gene of ribosomal RNA. Kinyoun's technique was detected positive in 8.1% (18/222), and PCR amplification was observed in 48.2% (107/222) of samples, which 63 were sequenced. Analysis of the sequences obtained showed that the most common species in these animals was *Cryptosporidium ryanae*, in buffalo calves after five days of age. The zoonotic species *Cryptosporidium parvum* were detected in only one animal and a genotype unusual, like *Cryptosporidium* sp. W20486 was first found in buffaloes.

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Host microtubule polyglutamylation is a critical tubulin post-translation modification in the invasion rate of *Toxoplasma gondii*

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Toxoplasma gondii, an obligate intracellular parasite belonging to the phylum Apicomplexa, is a major human and animal health concern. During the host cell invasion process, both the cell's and parasite's microtubule cytoskeletons present an active remodeling. Being so, proteins involved in the cytoskeleton remodeling and dynamics are excellent candidates to take part in the invasion process.
Katanin is a severing microtubule enzyme, a key player in cytoskeleton remodeling. Katanin's activity is selective, affecting different microtubule classes according to their post-translation modifications (PTMs) and inhibiting the accumulation of PTMs such as polyglutamylation. In fact, we observed in Katanin depleted RPE-1 cells a substantial increase of polyglutamylated microtubules. Furthermore, Katanin depletion also lead to abnormal centriole number, multipolar mitotic spindles and cell cycle arrest, illustrating the importance of this protein in microtubule dependent cellular processes. Polyglutamylation is described to reduce microtubule dynamics. Thus we investigated the ability of *Toxoplasma gondii* to invade host cells, in a scenario of low Katanin levels. Interestingly, the parasites have more difficulties to invade Katanin RNAi host cells than to invade control cells, probably due to the accumulation of polyglutamylated microtubules. To test this hypothesis, we overexpressed in RPE-1 cells two glutamylases (tubulin tyrosine ligases-like), and then analysed for *Toxoplasma gondii* invasion efficiency. As we expected, in these cells the parasites present decreased invasion efficiency in comparison to control cells. To confirm the involvement of microtubule polyglutamylation in Toxoplasma host invasion we are now investigating the impact of overexpressing a deglutamylation enzyme, a cytosolic carboxypeptidase, in the invasion process. Together our data strongly support that microtubule polyglutamylation being critical in the regulation of microtubule dynamics is a key factor during *Toxoplasma gondii* host invasion.

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The role of kinases, dynamin and actin inhibition at *Toxoplasma gondii* egress

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The apicomplexan parasite *Toxoplasma gondii* invades virtually all nucleated cells of warm-blooded animals. After multiplication inside a parasitophorous vacuole, which confers evasion from the host immune system, egress from host cell should occur and new neighbour cells can be invaded, spreading the infection. In order to study some of the processes involved in *T. gondii* egress we used calcium ionophore to synchronously trigger egress after treatment with either kinase, dynamin and actin inhibitors. Although parasite egress induction was only slightly affected by wortmannin and staurosporin treatment, the addition of genistein specific inhibitor of tyrosine kinase efficiently blocked the exit of parasites by more than 50%. The actin polymerization inhibitor cytochalasin D also blocked the induced egress of *T. gondii* and this blocking was further investigated by labelling host cell actin cytoskeleton. Fluorescence microscopy, however, showed parasites escaping preferentially in sites poor in actin filaments, indicating that host cell actin cytoskeleton integrity may be necessary for the parasite to migrate towards the site of egress. On the other hand, dynasore, which is known to inhibit GTPase activity of dynamin, had little or no effect on this step of the *T. gondii* cellular cycle. Taken together, these data indicate that egress involves multiple signalling routes and the direct interaction between the parasites and host cytoskeleton.

Characterisation of a cysteine protease expressed by *Eimeria tenella* and identification of its post-traductionnal regulator

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Cysteine proteases of the papain family are major virulent factors expressed by protozoa. They have been involved in many steps of parasites life cycle like cell invasion, intracellular replication, gametocyte formation and parasite differentiation. Their multiple roles in key steps of parasites biology make them attractive new therapeutic targets. Using BlastP, we identified 5 genes encoding for cysteine proteases in the genome of *E. tenella*. We named them *Eimeripain, EtCPL, EtCPC1, EtCPC2* and *EtCPC3* encoding respectively for one cathepsin B, one cathepsin L, and three cathepsin C. Complementary approaches of molecular biology and biochemistry revealed that most of these proteases are highly expressed and active in the unsporulated oocysts, suggesting a role in sporulation and/or gametogenesis. *Eimeripain* is the only activity that persists throughout the life cycle. We show that a specific inhibitor of Human cathepsin B, CA074-ME, inhibits Eimeripain and affects the capacity of sporozoites to invade MDBK cells. These data suggest that Eimeripain plays a central and pleiotropic role in *Eimeria* life cycle. Cysteine protease inhibitors from the Chagasin family are proteins expressed by protozoa that specifically bind to and inhibit cysteine cathepsins. As such, they participate to parasite pathogenesis. We identified a cysteine protease inhibitor, Eimestatine, expressed by *E. tenella,* which specifically inhibits the activity of Eimeripain in biochemical assays. Preliminary data suggest that Eimestatine forms a complex at each life stage, which may indicate a tight regulation of Eimeripain throughout the infectious process.

Besnoitia besnoiti and *Toxoplasma gondii*: different strategies to hijack the microtubule cytoskeleleton and Golgi apparatus of the host cell

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Besnoitia besnoiti and *Toxoplasma gondii* interact with the host cell microtubule cytoskeleton, not only during the first steps of host cell invasion, but also during parasite replication, since host cell microtubules around the established parasitophorous vacuole are constantly observed. This interaction implicates the recruitment of the host cell centrosome, the primary microtubule organizing center, at 18h after invasion by *T. gondii*, but not by *B. besnoiti*. Moreover, in cells overexpressing TBCCD1 (protein involved in nucleus-centrosome connection) the recruitment of the centrosome by *T. gondii* is less efficient to and the *T. gondii* replication rate is decreased. In *B. besnoiti* these differences were not observed. Given these results, the importance of the centrosome in cell migration, and the capacity described for *T. gondii* to modulate the motility of invaded cells, we studied the impact of these two parasites in host cell migration by wound-healing assays. We observed that cells invaded by *T. gondii*, but not those invaded by *B. besnoiti*, present a delay in wound closure. This is in agreement to the observed differences in centrosome recruitment.

Considering the close relation between the centrosome and Golgi apparatus, we have also assessed Golgi positioning. Surprisingly, in cells invaded by *T. gondii* and *B. besnoiti*, Golgi is consistently around the parasitophorous vacuole since 6h after invasion (one parasite/vacuole). However, in *T. gondii* invasion, Golgi ribbon is completely fragmented and close to the parasitophorous vacuole, while in *B. besnoiti* is intact.

In conclusion, *B. besnoiti* and *T. gondii* invasion requires the recruitment of the Golgi apparatus in completely different ways. Only *T. gondii* seems to recruit the host cell- centrosome which may be related to the recruitment of Golgi. On the other hand, *B. besnoiti* is able to directly recruit Golgi, even when it is apparently disorganized without requiring the recruitment of the centrosome. This reflects the two distinct evolutionary invasion mechanisms used by the two parasites.

Congenital toxoplasmosis in experimentally reinfected ewes

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The aim this study was evaluate the congenital transmission in experimentally reinfected and infected ewes, by oocysts *T. gondii*, in three pregnancies phases. Twenty ewes, negative serologically for *T. gondii*(IFAT-IgG), were selected and experimentally infected with ME49 strain(Day0). Three ram, negative serologically for toxoplasmosis, neosporosis, leptospirosis and brucellosis were used for natural mating. After the diagnosis of pregnancy, these ewes were distributed in four experimental groups: GI-five ewes reinfected with *T. gondii* on the 40th day of gestation(DG), GII-five in the 80th DG, GIII-DG 120th in five and GIV-five received saline solution in 120th DG(unreinfected). Five ewes, negative serologically (IFAT<64) for *T. gondii* infection were kept as negative control-GV. Seven days before the first infection, immediately prior to inoculation, every three days until the 30th day after inoculation and every seven days until the end of pregnancy, clinical examinations and blood samples(IFAT-IgG) were performed in 25 ewes. Ultrasonographic examinations were performed in the diagnosis of pregnancy and fortnightly after reinfection. Serum samples, from all the lambs were obtained immediately after birth(pre-colostral), at 3 and 14 days of life, for *T. gondii*(IFAT-IgG). Parasitism by *T. gondii* was investigated(histopathology, mouse inoculation and PCR) in tissue fragments of female and fetuses, stillbirths and/or dead lambs after birth. Twenty ewes showed *T.gondii* antibodies specific on post-inoculation day(PID) 11. The most serological title(2048) occurred 28 days after reinfection. All ewes produced lambs positive serologically for *T. gondii*. In groups I, II, III and IV were diagnosed reproductive disorders, such as birth defects, stillbirths and weak lambs. Some lambs that came forward had severe locomotive disorders. The results of the bioassay in mice and PCR revealed the presence of *T. gondii* in all 20 sheep and their lambs. Therefore, showed the congenital transmission of *Toxoplasma gondii* associated with reproductive disorders in sheep only infected and in ewes infected and subsequently reinfected by this protozoan.

A consecutive infection with two *Toxoplasma gondii* strains can affect the parasitic load in tissues of experimentally infected pigs

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One of the major routes for humans to get infected by *Toxoplasma gondii* is the consumption of raw or undercooked meat. In the present study, we compared the parasitic load in the tissues of pigs at slaughter age induced by the consecutive infection with 2 different *T. gondii* strains.
Four groups of three 6-week-old weaned piglets were orally infected with 6000 *T. gondii* tissue cysts as follows: Groups G and L were only infected once with the *IPB-Gangji* strain or the *IPB-LR* strain (4700 cysts) respectively; Group G/L was first infected with the *IPB-Gangji* strain and 2 month later with the *IPB-LR* strain; Group L/G received both strains in the inversed sequence. As negative control we kept one pig non-infected. All infected animals seroconverted. At 4 months p.i. the pigs were euthanized and the parasitic load was determined by qPCR in the following samples: brain, heart and 5 skeletal muscles, such as diaphragm, tenderloin and ham.
The hearts of all infected animals tested positive for *T. gondii* by qPCR, as did the brains with the exception for those from the Group G, which were all negative. The pigs in Group L had the highest parasitic loads and all tested tissues harbored parasites. Overall, all the animals who received the *IPB-Gangji* strain had lower parasitic loads or were even negative in some of their tissues. Group G had the lowest numbers of parasites and no parasites were detected in the brain, diaphragm, the ham and tenderloin.
Our study suggests that a consecutive oral infection in pigs with two different *T. gondii* strains can influence the quantity of cysts in their tissues. The *IPB-Gangji* strain could induce or fasten the clearance of infected muscle tissues in pigs, even of tissue cysts already present due to a prior infection, and thus possibly lower the infectiosity of meat.

Using magnetic capture and real-time PCR for detection of *Toxoplasma gondii* in tissue samples of experimentally infected goats and pigs

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Toxoplasma gondii infections are widely distributed in humans and in many warm-blooded species. One of the most common sources of *T. gondii* infection in humans is ingestion of undercooked meat containing tissue cysts. The standards for detecting *T. gondii* in meat samples are bioassays, but they are not applicable for screening large numbers of samples. Other preventive tests for detection of the contamination level of different types of meat are still missing due to lack of appropriate methods for detection of *T. gondii* in tissue samples. Magnetic capture (MC) is a new molecular method enabling detection of *T. gondii* in a large tissue sample and, in combination with qPCR for the 529 bp repeat element, allows quantification of *T. gondii* DNA concentration. In comparison with conventional methods of DNA isolation utilizing maximally 50 mg tissue samples, MC handles up to 100 g of the tissue. The aim of this study was to determine *T. gondii* distribution and predilection sites in food animals (goats and pigs) after experimental infection using MC qPCR technique. Goats were administered with 20000 oocysts p. o., pigs were administered with 5000 oocyst p.o. using the tiger isolate, genotype II. Goats euthanized at day 30 and day 90 after infection, and pigs euthanized at day 76 after infection were used in this study. Twenty to hundred grams of brain, lung, heart liver, spleen, kidney, both fore limbs, both hind limbs and dorsal muscles were tested using MC and qPCR. The difference of contamination level in tissues and the variance between two groups of goats was compared using a Man-Whitney test. In goats, lungs and brain were identified as the *T. gondii* predilection sites with highest *T. gondii* concentrations while in pigs it was the brain. A significant increase of *T. gondii* bradyzoites in goats 30 days post infection compared to 90 days post infection was revealed only in liver and dorsal muscle tissue. Our results confirm the suitability of MC qPCR method for the detection of *T. gondii* in tissue samples. Furthermore, we conclude that MC qPCR is suitable method for the assessment of the distribution of the tissue cysts of *T. gondii* and quantitative determination of *T. gondii* predilection sites in food animals.

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Stereological investigation of *Toxoplasma gondii* infection on mice kidney

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The intracellular parasite *T. gondii* is associated with morbidity and mortality for immunocompromised patients, including those submitted to organ transplant. In order to investigate if the infection of *T.gondii* promotes alterations on the number of nephrons in mice. sixteen female Swiss mice (21-24 g) were inoculated by gavage with 20 cysts of *T. gondii* ME49 strain. Six animals were killed after 25 days while ten were killed 57 days post infection. Other animals were used as controls. At the end of the experiment the animals were weighted and both kidneys were removed, dissected, measured and formalin fixed. Renal fragments were processed using routine histological methods and stained with hematoxilin & eosin. The number of glomeruli was calculated by stereological methods, based on the evaluation of renal volume, cortical-to-medullar ratio, glomerular volume density and volume weighted glomerular volume. Student´s-t-test was applied for mean comparisons, considering $P<0.05$ as significant**.** We observed that both groups of infected animals had 30% lower body mass when compared to controls ($p<0.001$). Also, the kidney volume was statistically reduced in infected animals. Again, this difference was found in the two groups of infected mice in comparison to controls ($p<0.05$). No significant differences in the cortical-to-medullar ratio, glomerular volume density and volume weighted glomerular volume were found. However, comparing to control animals, the number of nephrons was reduced by 23% and 28% in the infected mice after 25 and 57 days, respectively ($p<0.05$). These observations indicate that the oral infection of mice with *T. gondii* ME49 strain promotes major renal changes, resulting in the loss of nephrons.

Parasitological and pathological findings for the reproductive tract of bulls experimentally infected with *Neospora caninum*

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The objective of the present study was to investigate the presence and distribution of *Neospora caninum* and the associated histopathological findings in the genital tract tissues of bulls after experimental infection. Twenty-eight young bulls (1.5-2 years old) of the Asturiana de los Valles breed that were seronegative for *N. caninum* were used. Seven groups of four bulls (1 control + 3 experimentally infected) were slaughtered at 7, 14, 22, 29, 36, 41 and 78 days after infection with 10^8 tachyzoites of the Nc-1 isolate administered intravenously. Tissue samples (brain and genital tract) were aseptically recovered from each animal after slaughter for parasitological and pathological studies. The presence of *Neospora* DNA was determined using a nested PCR. Samples for pathological studies were processed with routine techniques. Immunohistochemistry was carried out on nested PCR positive tissue sections by means of an avidin-biotin-peroxidase technique. At 7 and 14 days after infection, *Neospora* DNA was found primarily in accessory gland samples. Later, the parasite was consistently found in the epididymis and testicles. *Neospora* DNA was found in brain samples at 22 days after infection. In all experimentally infected bulls the histopathological findings were focal or multifocal (perivascular and interstitium) aggregates of round cells, primarily lymphocytes and plasma cells, in the testicles (50%), epididymis (60.7%), accessory glands (21.4%), and penis (7.1%). The observed brain lesions were perivascular cuffs, meningitis and glial nodules. The main injuries were observed in the animals during the acute phase of neosporosis. Immunohistochemical detection of *N. caninum* revealed positive reaction in some testicle and brain samples with inflammatory lesions. The association between the presence of lymphoplasmacytic aggregates and positive PCR results had low concordance (kappa-value=0.104). In conclusion, young bulls experimentally infected with *N. caninum* showed the presence of protozoa and mild inflammatory lesions in the epididymis, testicles and accessory glands during the acute and chronic phases of infection.

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Characterization of the immune cell infiltration of cattle and buffalo placentas following experimental inoculation with *Neospora caninum* during early pregnancy

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Despite *Neospora caninum* (NC) being a major cause of bovine abortion worldwide, its pathogenesis is not completely understood. NC stimulates host inflammatory cell-mediated immune responses, which may be responsible for placental damage leading to abortion. Susceptibility of water buffalo (*Bubalus bubalis*) to NC is not well understood, although vertical transmission and foetal death has been confirmed after natural and experimental infections. The aim of our work was to characterise and compare immune responses in placental tissue following experimental infection in both species at 70 days of gestation. Cows and water buffaloes were infected with NC at day 70 of pregnancy and culled at 28 days post inoculation. Placentomes were examined by immune-histochemistry using antibodies recognising T-cell subsets (CD3, CD4, CD8, γδTCR), NK and B cells. Foetal death was confirmed in 3 out of 4 infected cows and 1 out of 3 inoculated buffalo. NC presence was confirmed in placental or foetal tissues using in 3 out of 4 infected cows, and in 3 out of 3 infected buffalos. Placental inflammation in NC-infected cows was generally moderate to severe, being more significant in the aborted animals. The inflammation in the buffalo placentas was scarce except for the case with the dead foetus where the inflammatory infiltrate was severe. In both species, cellular infiltrates were mainly characterised by the presence of $CD3^+$, $CD4^+$ and γδ T-cells; whereas $CD8^+$ and NK cells were less numerous. The distribution of the different cellular subsets observed in cattle and buffalo placentas was similar. In both species the infiltrates were more severe in the dams carrying dead foetuses. In general, cellular immune infiltrates in the placentomes were less severe in buffaloes, which may explain the lower number of abortion observed in this species after infection during early gestation.

Neospora caninum infection during early pregnancy in cattle: Influence of the isolate on abortion timing and immune responses

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The pathogenesis of abortion caused by *Neospora caninum* is complex, and different factors determine the outcome of infection, including gestation duration and foetal immunocompetence. Studies focused on the potential influence of the specific *N. caninum* isolate on abortion are very limited. In this work, we investigated the role of *N. caninum* intra-species diversity on the abortion outcome in cattle. Cows were intravenously inoculated at day 70 of pregnancy with 10^7 tachyzoites of two isolates that show marked differences in virulence *in vitro* and in mouse models: Nc-Spain7 (group 1, n=6), a high virulence isolate, and Nc-Spain8 (group 2, n=6), a low-to-moderate virulence isolate. Control cows (n=5) were inoculated with Marc-145 host cells. After inoculation, pregnancy was monitored, and dams were culled when foetal death was detected. Abortion occurred in all infected cows between days 24 and 49 post-infection; however, abortion occurred sooner in group 1 (median abortion day= 34) than in group 2 (median abortion day= 41). Similar histological lesions were found in all placentas (cotyledons and caruncles) and in most of the foetuses from the two infected groups. However, parasites were more frequently detected in the placenta and foetal tissues by PCR and in the brain by immunohistochemistry in group 1. Specific antibodies were detected from day 15 p.i. in all infected cattle, with a trend towards higher IgG levels in group 1. Differences in the IFN-γ and IL-4 secretion profiles were also found between infected groups in the lymphostimulation assays. Both infected groups showed significant increases in the levels of cytokine mRNAs (IFN-γ, IL-4, IL-10, IL-12p40 and TNF-α) produced in the placenta, and higher levels were found in the caruncles than in the cotyledons. Differences were also found between the infected groups: the IFN-γ levels were significantly increased in the caruncles of group 1, whereas higher IL-10 levels were detected for group 2.

The dense granule protein GRA9 in *Neospora caninum* and its characterization

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Several years ago we identified the dense granule protein GRA9 in *Toxoplasma gondii* (*Tg*GRA9) and we were interested if there exists a homologous GRA9 in the closely related parasite *Neospora caninum*. We were able to show that there is a putative homologous protein in *N. caninum* which seems to be a dense granule protein.
Immunofluorescence analysis of extracellular *N. caninum* tachyzoites revealed a dotted anti-*Nc*GRA9 staining distributed all over the parasite which suggested that the protein is located in the dense granules. Furthermore, *Nc*GRA9 was identified as one of the excreted secreted antigens (ESA) of *N. caninum* to which the dense granule proteins usually belong to. After invasion of the tachyzoites into their host cells *Nc*GRA9 was secreted into the parasitophorous vacuole (PV) where it targets to the vacuolar space and the PV membrane. Altogether, these properties of the protein imply that *Nc*GRA9 belongs to the dense granule proteins of *N. caninum*.
Fractionation analysis of extracellular *N. caninum* tahcyzoites revealed that *Nc*GRA9 remains as a soluble protein in the dense granules and is also present in association to aggregates in the dense granule core. After infection, *Nc*GRA9 is targeted into the PV and seems to be phosphorylated during or after secretion into the PV. Fractionation of infected cells by ultracentrifugation showed that *Nc*GRA9 in the PV is found in the soluble and the pellet fraction which indicated that the protein is at least partially associated to membranes within the PV either directly or indirectly by protein interactions.
In summary, our data show that the identified protein *Nc*GRA9 is a homologue of the already described *Tg*GRA9 and that *Nc*GRA9 belongs to the group of dense granule proteins of *N. caninum*. The characterization of *Nc*GRA9 demonstrated many similarities to *Tg*GRA9 which reflects the close relationship between the two parasites.

Specific antibody responses against *Neospora caninum* recombinant rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 proteins are correlated with virulence in mice

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The intraspecific diversity *Neospora caninum* is a determinant of *in vivo* parasite virulence and *in vitro* parasite behaviour. The relationship between isolate virulence and specific antibody responses targeting parasite key-proteins has not been thoroughly investigated. Thus, the kinetics and differences in the specific anti-rNcGRA7, anti-rNcSAG4, anti-rNcBSR4 and anti-rNcSRS9 antibody levels in groups of mice inoculated with ten different *N. caninum* isolates that differ with respect to virulence were analysed. The majority of virulence parameters analysed were correlated with specific antibody levels against the four recombinant proteins. First, the levels of antibodies developed against the highly immunogenic dense-granule protein NcGRA7 were significantly higher in mice inoculated with high virulence isolates than in mice inoculated with low-to-moderate virulence isolates in both the non-pregnant and pregnant mouse models. Moreover, these levels were correlated to anti-*N. caninum* IgG1 and IgG2a responses and the *in vitro* tachyzoite yield at 56 h (TY_{56}). Second, antibodies directed against bradyzoite-specific proteins were not detected in the non-pregnant model. Seropositive mice were mostly found in the groups inoculated with high virulence isolates such as Nc-Spain 7, Nc-Spain 4H and Nc-Spain 5H in the pregnant mouse model, most likely due to parasite reactivation and exposure of these bradyzoite proteins to the immune system. In conclusion, NcGRA7 could be used as a serological marker of virulence. Moreover, specific antibodies to bradyzoite stage-specific proteins seem to be related to virulence.

Indoleamine 2,3-dioxygenase and guanylate-binding proteins are involved in immune defense against *Neospora caninum*

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Neospora caninum (*N. caninum*) is an apicomplexan parasite closely related to *Toxoplasma gondii*. In nature this parasite is found especially in dogs and cattle, but may also infect other livestock. As an obligate intracellular parasite, *N. caninum* growth is mainly controlled by the cell-mediated immune response. During infection the cytokine interferon-gamma (IFN-γ) plays a prominent role in regulating the growth of *N. caninum* in natural and also experimental diseases.

The present study indicates that the induction of the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) is responsible for the inhibition of *N. caninum* growth mediated by IFN-γ activated human and bovine fibroblasts and endothelial cells. This antiparasitic effect could be abrogated by the supplementation of tryptophan as well as by the IDO-specific inhibitor 1-L-methyltryptophan.

In addition, we found that IFN-γ activated murine cells are also able to restrict the growth of *N. caninum* but IDO was not involved in this activity. Detailed co-localization studies indicate that immunity-related GTPases (IRGs) like Irga6, Irgb6 and Irgd and also guanylate-binding proteins (mGBPs) were involved in the inhibitory effect. These data underline species-specific differences in the control of *N. caninum* by human and murine cells.

An *in vitro* model of neonatal porcine coccidiosis – *Isospora suis* in an epithelial cell culture system

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To gain knowledge about the interaction between parasites and their host cells animal models may not always be sufficient. A reproducible *in vitro* cultivation system in representative cell lines offers the possibility of research on cell-cell interactions – like invasion, evasion and defence mechanisms – and also on mechanisms of pathogenesis. Moreover, highly purified parasitic material can be obtained and new drugs can be tested in advance to animal testing. Therefore, an *in vitro* system in a porcine epithelial cell line from the neonatal jejunum (IPEC-J2) was established for *Isospora suis*, an apicomplexan parasite causing neonatal porcine coccidiosis.

The establishment of the *in vitro* system included the setup of optimum purification procedures for oocysts, an excystation protocol, and culture conditions. Different infections doses and media compositions were tested to determine optimum culture conditions. Parasitic stages and host cell conditions were monitored semi-quantitatively and quantitatively. All developmental stages described for *in vivo* infections could be detected in the cell culture (meronts and merozoites of type I and II; micro- and macrogamonts and -gametes; oocysts). For harvesting merozoites, e.g. for use as antigen for stimulation assays of lymphocytes, an infection dose of 10:1 (IPEC:sporozoites) lead to an optimal recovery at dpi 5. Maximum densities of gametes were found with lower infection doses from dpi 9 on, a similar pattern was found for oocyst development.

Jejunal epithelial cells of neonatal piglets are the target cells of *I. suis*. Therefore, this system provides an appropriate *in vitro* model of neonatal coccidiosis, a disease with significant economic impact in swine production. At the moment first attempts are made to investigate the innate immune response to the infection and antigen-presentation on the level of the epithelial host cells. Findings from this cell culture system may also give input for further development of *in vitro* models for avian coccidiosis.

Early response of epithelial cells to *Eimeria tenella* infection

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Eimeria tenella infection is associated with a severe intestinal disease leading to high economic impact in poultry industry. The cost of vaccine and the emergence of anticoccidial drug resistances highlight the need of alternative strategies. For this purpose, the understanding of the cellular and molecular mechanisms involved in the development of the disease at the earliest time of the infection is needed. Our objective is to determine the early epithelial cell response to *Eimeria* infection. We developed an *in vitro* model of mouse intestinal epithelial cells (mICcL2) infected with *Eimeria tenella* on which a RNA sequencing study was performed. Based on a cut-off of >2 fold differential expression compared with the uninfected cells, early infection (1-4h) with *Eimeria tenella* leaded to less than <1% changes in gene expression. Out of 76 genes whose expressions were modified 1h pi, 25 were upregulated, in contrast to 58 out of 78 4h pi. Transcription factor genes and genes related to the immune response were highly upregulated 1h pi whereas transcription factor and immune response genes but also genes encoding for cytoskeletal and adhesion molecules were upregulated 4h pi. Interestingly, the expression of the transcription factors fosB and c-fos was the most induced by the infection (30 and 19 fold after 1h). The Fos family transcription factors is implicated in many cellular functions such as cell cycle, differentiation and immune response and is upregulated in different models of infection. The role of cFos in an *Eimeria* infection of epithelial cells will be investigated using a siRNA approach.

The effect of Eimeria co-infection on *Campylobacter* colonisation of chickens

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The Apicomplexan parasite *Eimeria* causes the disease coccidiosis. All livestock are likely to be affected by coccidia, most notably poultry. While the impact of coccidia on the poultry industry is well recognised in terms of direct pathogenicity, the influence of *Eimeria* infection on the enteric microbiota is an area that remains largely unknown with the possible exception of *Clostridium perfringens*. It is clear that the gut microbial population is important in maintaining metabolic efficiency and has a role in protecting against pathogen colonisation. The importance of a balanced microbiota indicates a broader impact of eimerian infection, even when disease is sub-clinical.

Campylobacter is the most common cause of bacterial food poisoning in humans in the developed world and has been implicated as an infectious pathogen of poultry. Due to the zoonotic potential of this bacterium, coupled with the economic impact on food production and issues of animal welfare, *Campylobacter* is of great sociopolitical importance. Nonetheless, the influence of the enteric microbiota on *Campylobacter* colonisation within the avian intestine and deeper tissues remains a neglected area of research. Quantification of early *Campylobacter jejuni* colonisation of the chicken caeca, liver and spleen revealed significant variation in the presence of concurrent *Eimeria tenella* infection. Intriguingly, parasite co-infection was associated with an elevated *C. jejuni* load within the caecal lumen three days post bacterial challenge but reduced translocation to the liver and spleen. Thus, while faecal shedding of *C. jejuni* may be at least temporarily increased by overlapping *E. tenella* infection, deep tissue bacterial contamination may be decreased. These studies may have an impact on the development of *Eimeria* species as vaccine delivery vectors as eimerian co-infection has been shown to influence *Campylobacter* colonisation in poultry. For *C. jejuni* the public health risk associated with contaminated chicken liver may promote their use.

Chronic bovine besnoitiosis: histopathological findings and parasite distribution and load in subclinical cases

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Bovine besnoitiosis, caused by *Besnoitia besnoiti,* is a chronic and debilitating disease. The most characteristic clinical signs of chronic besnoitiosis are visible tissue cysts in the scleral conjunctiva and the vagina, thickened skin, and a generally poor body condition. However, many seropositive animals remain subclinically infected, and the role that these animals play in spreading the disease is not known. The aim of the present study was to assess the serological status, tissue distribution, and parasite load of subclinically infected animals. Histopathological, immunohistochemical and molecular analyses were performed using several tissues from the respiratory and reproductive systems, in addition to other internal organs and skin, from six cows that had exhibited scleral cysts and specific antibodies in the past but did not show any clinical signs at the time of slaughter. Tissue cysts were located primarily in the upper respiratory tract, i.e., the rhinarium and larynx/pharynx, were found in 4 cows. The next most common cyst locations were the distal genital tract (vulva/vagina) and the skin of the neck, found in 3 and 2 cows, respectively, out of the 4 cows showing cysts in the respiratory tract. We were unable to detect any parasite in the remaining 2 cows. Tissue cysts were located in the conjunctive tissue, and in two cows, these cysts were associated with a non-purulent inflammatory infiltrate consisting primarily of T lymphocytes. The correlation between the histopathological results and PCR was very good, although the latter was moderately more sensitive. The parasite burden, estimated based on the number of cysts and the quantitative real time-PCR results, was very low. It is noteworthy that the only animal that showed a recent increase in seropositivity showed the highest burden and the most conspicuous inflammatory reaction against the cysts.
In conclusion, although these cows no longer displayed any visible signs of besnoitiosis, they remained infected and therefore may still be able to transmit the parasite.

Experimental infection of dogs and gerbils with *Hammondia heydorni*

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This study aimed to isolate and induce experimental infections in dogs and gerbils with *Hammondia heydorni*; observing clinical, parasitological and histopathological features. The inoculates were recovered from naturally infected dogs in the city of Campo Grande, MS, Brazil; 969 stool samples from dogs were examined, 17 of which (1.75%) had oocysts of the *Neospora*-like protozoan. Of these, five were confirmed by PCR as *H. heydorni*, producing amplified fragments of approximately 270 bp. Eight gerbils were inoculated, using the samples confirmed by PCR. Simultaneously, seven dogs were kept in isolation from birth. At the forty-fifth day of age they were divided into two groups: one with five (IG) and the other with two animals (CG). The gerbils were necropsied at 129 days after infection (DAI), and small pieces of organs and tissues were collected for later processing by PCR. To inoculate the dogs (IG) the carcasses and remains of the gerbil organs were finely chopped, homogenized and divided into individual portions of 94g each and offered to the animals (IG) after fasting. The dogs were necropsied at 10 and 16 DAI, and tissue samples were collected for histopathology, scanning electron microscopy (SEM) and PCR. Observations showed numerous bleeding spots in the jejunum and ileum, and the presence of mucus in all portions of the small intestine in three of the dogs from GI. Histopathology observed two dogs of the same group displaying desquamation at the intestinal villi and hypertrophy of the goblet cells. The SEM images of samples of the duodenum, jejunum and ileum of group GI showed the destruction of the villi in the intestinal mucosa. The DNA fragment was amplified from lung and mesentery of infected dogs and gerbils, using primers designed for the amplification of a 270-bp *H. heydorni*.

Searching for Plk1 interaction partner on the surface of *Theileria annulata*

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Sporozoites of the bovine parasite *Theileria annulata* infect monocytes/macrophages and B cells. After entering the cell, the sporozoites escape the parasitophorous vacuole and associate with the host cell microtubules (MTs). The parasite differentiates into a strictly intracellular macroschizont and induces transformational changes in the host cell. Transformed cells become resistant to apoptosis and undergo uncontrolled proliferation, making their behavior comparable to that of cancer cells. Parasite division is a passive process, and relies upon host cell cytokinesis in these continually dividing cells. During mitosis and cytokinesis (M-phase) schizonts associate closely with astral and central spindle MTs, resulting in the schizont being equally distributed between the two daughter cells. The association of the parasite with central spindles was shown to be dependent on host cell Plk1 (polo-like kinase 1) activity. Plk1, a serine-threonine kinase, is an important regulator of cellular operations during M-Phase and was shown to be recruited to the parasite surface in a cell cycle-dependent manner (von Schubert et al., 2010). However the detailed mechanism by which recruitment to the parasite occurs, and the binding partner of Plk1 on the schizont surface, are still unknown. The identification of the Plk1 binding partner is currently underway. A protocol using nocodazole and a Nycodenz gradient has been established in which schizonts, together with bound proteins, can be isolated from *Theileria*-infected macrophages (TaC12 cells) in large quantities. The liberated parasites are subsequently incubated with a broad range crosslinker, in order to stabilize the interaction of Plk1 to the binding-partner, and subjected to immunoprecipitation using ant-Plk1 antibodies. Conditions are currently being optimized to identify binding proteins or cross-linked Plk1 complexes.

Characterization of a putative secreted patatin-like phospholipase

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Theileria spp. infect leukocytes and have the unique ability to convert the host cell into a so-called transformed stage, conferring uncontrolled proliferation and resistance to apoptosis. Proteins that are expressed on the parasite surface or secreted into the host cell cytoplasm are the most likely candidates to be involved in host cell transformation. Unlike several other apicomlexan parasites, *Theileria* lives free in the cytoplasm, which is advantageous in terms of potential to directly interact with and modify the host cell. Shortly after the parasite enters the host cell, the surrounding host cell plasma membrane is destroyed. This is of critical importance because parasites unable to degrade the membrane cannot survive. One interesting candidate with the potential to destroy the surrounding plasma membrane is a *Theileria*-encoded patatin-like phospholipase. While this protein has a predicted signal peptide, whether it really is secreted is not known. Therefore, an initial set of experiments was carried out to assess the functionality of the signal peptide in an *in vitro* translation and transcription system, in the presence or absence of microsomes. Antibodies will be produced and localization experiments performed in order to assess whether the protein is expressed by the sporozoite and secreted into the cytoplasm of the host cell. Finally the ability of this phospholipase to destroy the plasma membrane will be tested by expression in *Toxoplasma gondii*.

An outbreak of toxoplasmosis in rabbits in Argentina

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Toxoplasma gondii is a protozoan parasite that affects domestic and wild animals. Toxoplasmosis is a world-wide distributed zoonosis. Rabbits are susceptible to this protozoan and may die by acute infections. The aims of this study were to identifiy *T. gondii* in tissues of suspected naturally infected rabbits and to characterize them through molecular methods. A sudden mortality outbreak was registered in an intensive rabbit farm of 300 mothers, located in the province of Buenos Aires, Argentina. Macroscopic lesions found in spleen were suggestive of toxoplasmosis. Samples (n=19) from central nervous system (CNS), liver, spleen and lung were examined by fresh observation, bioassays, histopathology (HP), and polymerase chain reaction (PCR). Indirect fluorescent antibody test (IFAT) was performed on 12 tissue fluids samples. Spleen and SNC pools were homogenized and observed in fresh and 4 Swiss mice were inoculated. DNA was extracted from samples with a commercial kit and PCR was performed using TOX5-TOX8 as specific primers for *T. Gondii.* In addition, nSAG2, SAG3, BTUB, GRA6, c29-2, c22-8, L358, PK1 and Apico markers were evaluated by nested-PCR followed by restriction fragment length polymorphism analysis (PCR-RFLP).Tissue cysts were observed in the fresh spleen homogenate. Compatible toxoplasmosis lesions were observed in all organs, except lung, by HP. *T. gondii* specific antibodies were detected (≥ 1:40) in all rabbit fluids analyzed by IFAT. Three of 4 inoculated mice were seropositive by IFAT at dilutions 1:50, 1:200 and 1:800. Specific *T.gondii* DNA was characterized as genotype III for the nine markers. This genotype was previously isolated from domestic and wild animals of Argentina, including a previous outbreak of toxoplasmosis in rabbits in a farm in La Plata.

Immunoreactivity of sera from naturally and experimentally infected cows (Nc-6 Argentina, Nc-1) to *Neospora caninum* immunodominant antigens

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Neospora caninum is a protozoan parasite that causes abortion and important economic loss in argentinian cattle. The accurate diagnosis of *N. caninum* infection is essencial for control. The aim of this study was to determine and compare immunoreactivity of sera from naturally (NI) and experimentally infected cows (EI) (Nc-6 Argentina, Nc-1) to *N. caninum* immunodominant antigens by Immunoblot (IB) and Immunofluorescence Antibody Test (IFAT). Serum samples were obtained from NI (n=266), EI Nc-6 (n= 18) and Nc-1 (n=30) cows. Sera were analyzed by IFAT in two-fold dilutions and IB performed in non-reduced conditions with Nc-1and sera dilution of 1:100. Animals were classified as seropositive by IFAT ≥1:50 and IB whenever 2 or more immunodominant antigens (IDA) were detected. A very good agreement between IFAT and IB (k=0.85, p<0,001) was observed. Based on the frequency and intensity of recognition, five IDA (19, 29, 30, 33, and 37 kDa) were recognized by sera from all studied groups. IDA were recognised at high dilutions in most EI sera but also at low titres in NI cows. A 37 kDa and 29 kDa antigens were detected in 100% and 96% of seropositive animals, respectively. The 30 and 33 kDa antigens were recognised with higher frequency and intensity in IFAT samples with titres ≥1:400. The 17 kDa protein was only recognized in IFAT samples with titres ≥ 1:100 and a protein ~26 kDa was present in IFAT titres ≥ 1:3200. A clear relationship between increasing IFAT titre and more intense and frequent antigen recognition was observed. Similar immunoblotting patterns were observed in NI and EI cows. In conclusion, the 37 kDa and 29 kDa proteins are suitable antigens for immunodiagnosis of neosporosis in cattle. In addition, there were no differences in immunoreactivity of sera from cows infected with the studied isolates and those present in the field in Argentina.

Immunodominant antigens of *Neospora caninum* in experimentally infected water buffaloes (*Bubalus bubalis*)

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Neospora caninum is an Apicomplexan parasite related to abortion in beef and dairy cattle. Water buffaloes (*Bubalus bubalis*) are intermediate hosts for *N. caninum.* Around 80,000 heads of water buffaloes are raised under extensive conditions in wet areas of the northeast of Argentina and a seroprevalence for *N. caninum* of 64% has been reported. There is little information available for the diagnosis of neosporosis in wild species like water buffaloes , for that reason it is important to count with proper serological tests. The aim of this study was to identify antigens for immunodiagnosis of *N. caninum* based on the serological response from experimentally infected (EI) water buffaloes and to compare the immunoblotting pattern with EI cows. Two Mediterranean water buffaloes seronegative to *N. caninum* were inoculated intravenously with 10^8 tachyzoites of *N. caninum* Nc-1 strain. Blood samples were taken at days 0, 7, 14, 21, 28 postinoculation. Sera from EI cows intravenously inoculated with 10^8 tachyzoites of Nc-1 strain were also used. Indirect Fluorescence Antibody Test (IFAT) and Immunoblot (IB) with non-reduced antigen from Nc-1 and sera dilution of 1:100 were performed to all serum samples. Sera were classified as positive when IFAT titre ≥ 1:50 and 2 or more immunodominant antigens (IDA) were recognised. The main IDA detected in water buffaloes and cows were: 17, 29, 30, 33, 37 kDa proteins. There was no significant differences in the immunoblotting pattern of recognition in both species except for the presence of a ~26 kDa antigen detected only by cow sera with IFAT titres ≥ 1:3200 but not in water buffaloes with similar titres. Therefore, these 5 IDA can be used for immunodiagnosis of neosporosis in different species, as it has been probed to be recognised by sera from water buffaloes and cows under the same experimental conditions.

Blurred epidemiology of bovine besnoitiosis: parasite detection in skin among seropositive cattle

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Background: The life cycle of *Besnoitia besnoiti*, the agent of bovine besnoitiosis and the epidemiology of bovine besnoitiosis are not yet elucidated. Only a relatively small amount of infected cattle develop obvious clinical signs and most of them remain asymptomatic and seropositive for a long time. The cattle-to-cattle contamination is probably the most common way of infection via biting flies. According to this assumption, the identification and the selective culling of cattle having high concentrations of bradyzoïtes cysts in skin should be a major way of disease control. The aim of this preliminary study is to assess the proportion of animals showing positive PCR reactions in skin biopsies among seropositive ones.

Protocol: Blood and skin biopsies were sampled on 154 slaughtered cattle, from free and besnoitia-infected areas of France (64 and 90 animals respectively) to assess both serological status and presence of *B. besnoiti* DNA. Serological analyses were done by Western Blot (WB). Real time PCR (qPCR) tests were performed on skin samples of right neck taken from each animal by using the commercial PCR kit Adiavet™ Besnoitia.

Results and conclusions: All cattle from besnoitiosis free area were negative in serology. Among them, 63/64 were qPCR-negative. Coming from the infected area, 36/90 animals were tested WB-positive and within this WB-positive group, 16 animals were skin qPCR-positive. Among the 54 WB-negative cattle from the infected area, 50 were also qPCR-negative. Then, only 44.5% of samples were positive for the both analyses in the infected area. Positive Ct values in skin biopsies ranged from 20 to 39 with a median Ct value of 35.5. Surprisingly, five cattle, whatever their origin, were found to be WB-negative and qPCR-positive with high Ct values (≥ 37) for four of them. Further studies are required to confirm the relevance of those various subsets and to assess their respective epidemiological role.

Cryptosporidiosis in cattle, buffalo and humans in the Ismailia province of Egypt: Epidemiology and molecular analysis

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In this study, prevalence of *Cryptosporidium* spp. in faeces from cattle, buffalo and diarrheic children (<10 years) in the Ismailia province, 120 KM east of Cairo, Egypt, was investigated. Respectively, 804 and 165 samples collected from animals and humans were first screened by the Copro-antigen *RIDA®QUICK* test. Positive samples as well as 10% of randomly selected negative samples were further tested by generic polymerase chain reaction (PCR) assays aiming at the partial amplification of the 18S ribosomal DNA gene and 60-kDa glycoprotein (GP60) encoding gene. At an estimated prevalence of approximately 43% in animals, about 66%, 12% and 4% were identified as *Cryptosporidium (C.) parvum, C. ryanae*, *C. bovis*, respectively using PCR and restriction fragment polymorphism analysis. Moreover, mixed infections of *C. parvum* with *C. ryanae* as well as *C. parvum* with *C. bovis* and *C. parvum* with *C. andersoni* were observed. On the other hand, out of 49% of positive human samples, 61%, 38% and 1% were identified as *C. hominis*, *C. parvum* and *C. parvum* plus *C. bovis,* respectively. Subtype family IId (mostly genotype IIdA20G1) predominated over IIa (genotype IIaA15G1R1) in animals while subtype families IIa (genotypes IIaA15G1R1 and IIaA15G2R1) and IId (genotype IIdA20G1 only) were equally identified in humans. There was no significant difference in prevalence of cryptosporidiosis among buffalo and cattle. Infections with the IId subtype family were predominant in animals younger than 3 months but *C. andersoni* occurred only in cattle older than 1 year. Conversely, cryptosporidia were not identified in toddlers younger than 7 months. Zoonotic transmission due to close contact with animals was a statistically significant risk factor of human infections; nevertheless other sources of infections have been discussed.

Molecular identification of *Cryptosporidium* from calves in Argentina

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Diarrheal disease is one of the main causes of morbility and mortality in cattle worldwide. *Cryptosporydium* sp. is one of the most common enteric pathogens in calves of 30 days or younger. In Argentina, it is known that cryptosporidial infection is responsible to significant economic losses in rearing calves. Nevertheless, molecular identification of *Cryptosporidium* sp. has been reported in a very few surveys.

The objective of the present study was to assess *Cryptosporidium* infections in neonatal calves by PCR and to carry out the genotyping. Five fecal samples submitted to Immunoparasitology Laboratory (FCV-UNLP) in October 2011, were collected from symptomatic calves less than 1 month of age from a farm of Buenos Aires province, Argentina. Samples were examined for the presence of *Cryptosporidium* sp. oocyst using a concentration method which combines flotation and sedimentation techniques and modified Ziehl Neelsen staining technique. Genomic DNA was extracted from *Cryptosporidium* sp. positives samples by a QIAamp stool Mini Kit (Qiagen) and amplified by nested polymerase chain reaction (nested PCR). PCR was performed with primers pairs targeting *Cryptosporidium* 18 S ribosomal RNA (18 S rRNA). Secondary PCR products were analysed on 1% agarosa gel and visualized by Sybr safe staining. Finally, these products were sequenced to confirm genotype identification comparing the sequences obtained with those registered in GenBank by BLAST analysis. Four specimens were positive for *Cryptosporidium* sp. by microscopy as well as by PCR technique. The isolates were identified as *Cryptosporidium parvum (C. parvum*). Results of the present study indicate that microscopy may be an useful tool for *Cryptosporidium* diagnosis in symptomatic calves, whereas molecular characterization is required to determine the risk of zoonotic infection on a farm.

Identification and analysis of candidate antigens for ELISA based diagnosis of *Theileria annulata* infections

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Tropical or Mediterranean theileriosis, caused by the protozoan parasite Theileria annulata, is still an economically important bovine disease in North Africa, Southern Europe, India, the Middle East and Asia. The disease affects mainly exotic cattle and imposes serious constraints upon both breed improvement programmes and livestock production, especially in developing countries. Diagnosis of T. annulata infection in cattle is based on three main principles; (a) detection of the parasite in Giemsa-stained lymph node biopsy smears or in peripheral blood smears, (ii) molecular diagnosis of amplified parasite DNA and (iii) serological tests that detect antibodies that react specifically against parasite antigenic proteins. In the present study, genes encoding candidate antigenic proteins were bioinformatically identified in the T. annulata genome sequence based on possession of SP, GPI anchor, TMD, $d_N d_S$ values and EST data. Bioinformatically identified candidate genes, were cloned and expressed as recombinant protein to evaluate immunogenic properties compared to previously identified antigens Tams1-2, TaSP, Tamr–1, NC-1, NC-10 and SPAG-1, using western blot and ELISA. Results obtained from this analysis indicated that bioinformatically identified protein candidates:TA06510, TA20440, TA13755, TA15690, TA15695, TA15705 (Ta9), TA15710 and previously identified proteins: TaSP, Tams 1-2, SPAG-1, HSP70 , NC-1, NC-10 and Mero I were all immunogenic. Western blot analysis also showed that two immunodominant proteins detected in D7 infected cell line extracts are represented by TA15705 and TA15710, but not the TaSP antigen that was thought to be immunodominant based on previous studies. The data obtained from ELISA indicated that due to either allelic sequence polymorphism or differential immune responses of individual animals, all of the recombinant proteins tested were considered not to be suitable for routine, robust diagnosis of tropical theileriosis in the field and that further work in this area is required.

Cloning and expression of SAG1 antigen from *Toxoplasma gondii* in fusion with the OprI bacterial lipoprotein, a TLR ligand

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The activation of pattern recognition receptors (PRRs) on antigen presenting cells (APCs) has a crucial impact on the development of adaptive immune responses. This fact has been extensively explored during the last years as a strategy for the development of novel subunit vaccines, namely through the conjugation of antigens with ligands for these receptors. Recently, we have developed a new expression system for the production of antigens in fusion with the OprI lipoprotein in *Escherichia coli*. OprI is a toll-like receptor (TLR) ligand naturally found in the outer membrane of *Pseudomonas aeruginosa* and we have shown that it has the typical structure of a TLR2/1 agonist when expressed in *E. coli*. Here, we report the cloning of the full sequence of *Toxoplasma gondii* surface antigen SAG1 in the newly developed vectors pOL and pOLM and its expression in fusion with OprI. The production of lipidated (pOL) and non-lipidated (pOLM) fusion proteins was demonstrated and the translocation of the OprI-SAG1 lipoprotein to the outer membrane of the *E. coli* expression host was also confirmed. A relevant impact of OprI-SAG1 lipoprotein expression on the viability of the host cells was observed, underlining the advantage of the tight control over expression offered by this system. Purification of lipidated OprI-SAG1 by denaturing affinity chromatography is now being carried out using previously established protocols and native purification of the non-lipidated fusion protein will be attempted in order to obtain an appropriate control for immunization experiments. In the future, the profile of the immune response induced in mice by the inoculation of the lipidated and non-lipidated OprI-SAG1 fusion products will be addressed and their potential use in challenge experiments with *T. gondii* will be evaluated.

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Development of inactivated *Neospora caninum* vaccines based on nano/microparticles

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Many efforts are being carried out to develop a safe and effective vaccine for the control of neosporosis. The use of innovative adjuvants that can boost parasite antigen immunogenicity and induce an appropriate immune response is a critical factor in designing inactivated formulations. Nano/microparticles could be an excellent vehicle and a potent adjuvant for killed vaccines formulations due to their ability to deliver and gradual release of their cargo. The objective of this study was to evaluate the safety of and induction of immune responses by inactivated vaccines encapsulated in nano/ microparticles in a mouse model. *Neospora caninum* antigen extract (TEX) and lyophilised tachyzoites (LTZ) were encapsulated in PLGA and Gantrez nanoparticles and in poly-caprolactone (PCL), PLGA and Zein microparticles, respectively. The efficiencies of entrapment were greater than 60% in all cases. Groups of BALB/c mice were immunised subcutaneously three times at three-week intervals, and both humoral and cellular immune responses were evaluated. Immunisations did not produce local or systemic reactions. All formulations induced high levels of anti-*Neospora* IgG1 antibodies, and higher values were observed in the PLGA/TEX, PCL/LTZ and TEX groups ($P<0.001$). The production of IgG2a was detected only in mice immunised with Gantrez/TEX, PLGA/LTZ, TEX and LTZ, and the TEX group produced significantly higher levels of IgG2a ($P<0.001$). A cellular immune response was observed only in these groups. The PLGA/LTZ, LTZ and TEX groups showed high levels of IFN-γ and detectable IL-4 production ($P<0.05$). The highest IL-4 values were detected in the PLGA/LTZ group ($P<0.05$). Taken together, our results indicate that LTZ and TEX are valuable antigens for use in inactivated vaccines because balanced Th1/Th2 immune responses were observed. All of the nano/ microencapsulated formulations triggered a strong humoral immune response in terms of IgG1, but only the combination of PLGA and LTZ significantly induced the cellular immune response. Further studies are necessary to determine the protective efficacy of these vaccine products.

IFN-γ mediated immune response elicited in the intestinal mucosa of mice infected intragastrically with *Neospora caninum*

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Horizontal transmission through the ingestion of sporulated oocysts significantly contributes for the high prevalence of neosporosis in cattle. Therefore, the local immune response in the intestinal mucosa may be a privileged form of the host to counteract or avoid infection. As the Interleukin-12/Interferon-γ axis is essential for immune protection against this parasitic infection, we assessed, in a murine model of intragastric (i.g.) infection with *N. caninum* tachyzoites (NcT), the production of these cytokines in the intestinal mucosa, mesenteric lymph nodes (MLN) and spleen. Soon after infection, NcT could be found in the intestinal lamina propria and MLN of C57/BL6 mice. Accordingly, MLN conventional and plasmacytoid dendritic cells displayed an activated phenotype 18h after the parasitic challenge, as assessed by upregulated surface MHC class II and CD86 expression, and had increased IL-12 production. The frequency of TCRβ $CD8^+$, but not of TCRγδ or TCRβ $CD8^-$ IEL, producing INF-γ^+ was increased in mice challenged i.g. with NcT, comparatively to mock-infected controls, 48h upon infection. Later in infection, $CD8^+$ INF-γ^+ T cells were found in increased frequencies in the MLN and spleen. No such difference was found in $CD4^+$ T cells. Altogether, our results show that protective cytokines IL-12 and IFN-γ are produced in the intestinal mucosa or associated lymphoid tissue early upon i.g. infection with NcT in C57BL/6 mice. These results obtained in the murine model, by showing that an IFN-γ mediated imune response is elicited in the intestinal mucosa by NcT, suggest that potentiating this response by mucosal immunization with *N. caninum* antigens may be worth to attempt as an alternative strategy towards vaccination against neosporosis in cattle.

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Protective effect of intranasal immunization with *Neospora caninum* membrane antigens against murine neosporosis established through the gastrointestinal tract

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Neospora caninum emerged in the past few decades as one of the main pathogens causative of economic loss in cattle industry. In the present study, we have evaluated the effectiveness of intranasal immunization with *N. caninum* tachyzoites membrane protein extracts as target antigens and CpG as adjuvant, in a murine model of intragastrically-established neosporosis. Nearly all of the immunized mice presented no detectable parasitic DNA in the liver or brain upon infection with 5×10^7 tachyzoites, indicating that robust protection was achieved with this immunization strategy. The immunization procedure elicited the production of antigen-specific IgA in the intestinal mucosa and IgG antibodies, detected in the serum. The isotypic profile of serum IgG antibodies indicated that a predominant Th1-type immune response was induced upon immunization. However, interferon-? was not detected in the immunized mice, above the control levels. Altogether these results show that mucosal immunization with *N. caninum* membrane proteins with CpG adjuvant prevents intragastrically established neosporosis in mice and indicate that a parasite-specific humoral immune response elicited locally in the intestinal mucosa may have a significant role in the protection of the host.

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Different outcomes of protection against *Neospora caninum* infection after vaccination with a chimeric antigen in the pregnant and in the non-pregnant mouse model

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Neospora caninum (Apicomplexa: Eimeriina: *Sarcocystidae*) is reported as the leading cause of bovine abortion, thus the disease represents an important veterinary health problem and is of high economical significance.
The overall goal of our investigations on *N. caninum* is to develop a vaccine that limits both the cerebral infection and the transplacental transmission. Since promising results were obtained with a combination of the recombinant forms of two microneme proteins, NcMIC1 and NcMIC3 and one rhoptry protein, NcROP2, in the reduction of cerebral infection and vertical transmission in infected mice (1), we focused on the use of these proteins for further vaccination strategies. We created four different chimeric proteins composed of their respective predicted putative antigenic domains placed in different order. BALB/c mice were vaccinated with the different antigens solubilised in saponin and challenged with $2x10^6$ *N. caninum* tachyzoites. One of the chimeric proteins, recNcMIC3-1-R, conferred significant protection against cerebral infection (2). A second experiment was performed with the protective antigen (recNcMIC3-1-R) in the pregnant mouse model. Mice were vaccinated, mated, and challenged at day 7-9 of gestation. However, no protection against transplacental transmission and against cerebral infection in the pregnant dams was observed in the vaccinated group. After challenge, the non pregnant mice showed a high IFN-γ/IL-4 ratio, while the pregnant mice showed an overall lower cytokine production with a higher IL-4/IFN-γ ratio. In order to counterbalance the strong Th2 response in the pregnant mice, a third vaccination trial employing Freund's incomplete adjuvant, a stimulator of cellular immunity, was planned and is currently in progress. A comparison of the degrees of protection achieved and the immune responses observed in the three models will be presented.

The adjuvant immune modulation effect in experimental vaccine against *Neospora caninum* using rNP43 expressed in *Pichia pastoris*

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The development of an effective vaccine against *Neospora caninum* infection in cattle is an important issue due to the significant economic impact of this parasitic disease worldwide. In this study, the immune response of different vaccine formulations using the *N. caninum* recombinant proteins NP43 (immunodominant tachyzoite surface protein) expressed in *Pichia pastoris* was evaluated. We investigated, by ELISA the IgG dynamics and by qRT-PCR cytokine expression patterns of different adjuvants used in vaccination. Mice (10/group) were immunized intra-muscular with 20 μg μL^{-1} of rNP43 with two doses twenty one days apart, alone or adjuvanted with oil, bacteria polysaccharide, and alumen hydroxide. The mice were bled every seven days and the serum separated for IgG ELISA evaluation. At 28 days, four mice of each group were euthanized, splenocytes cultured and stimulated with rNP43. Total RNA was extracting using Trizol and cDNA prepared. The results show higher antibodies titers in the oil, followed by the polysaccharide group. Also we observed an increased IgG2 modulation by the polysaccharide group. The rNP43 alone induced a significant high expression level of IL-17 (120 fold increase), that was significantly reduced by the association with the adjuvants oil, alumen hydroxide and polysaccharide in 100, 5 and 17%, respectively. The oil was able to up regulate TNF-α 5 fold, but none of the other cytokines studied (IFN-γ, IL-4, IL-10, IL-12), whereas the bacteria polysaccharide up regulated IL-4, IL-10, lL-12 by 58, 2 and 1.6 times respectively. Alumen hydroxide was able to up regulate IFN-γ, TNF-α, IL-4, IL-10, IL-12 by 6, 4, 9, 2 and 4 fold respectively. This study demonstrates the impact that adjuvants can have on the modulation of vaccine immune response against *N. caninum.*

Histopathological lesions in placenta and fetuses from heifers vaccinated with experimental vaccines and challenged with *Neospora caninum*

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The aim of this study was to characterize the histophatological lesions in fetuses from heifers vaccinated with native antigens and recombinant proteins from *Neospora caninum* formulated with immune stimulating complexes (ISCOMs). Twenty seronegative *N. caninum* pregnant heifers were involved: 4 were inoculated intravenously with $1x10^8$ live tachyzoites of Nc 6 strain (Argentine isolation) (Group A); 4 were inoculated with native antigens obtained from tachyzoites of Nc 6 strain (750ug/dose) formulated with ISCOMs (Group B); 4 were inoculated with a mix of four recombinant proteins (SAG_1, PIs, Hsp20, GRA7 (30ug of each protein/dose)) also formulated with ISCOMs (Group C); 4 received ISCOM-MATRIX (Group D) and 4 were controls receiving PBS (Group E). All the heifers of these last four groups were inoculated twice by subcutaneous via with interval of 21 days previous mating. After pregnancy was confirmed, all animals were challenged at day 70 of gestation with $4x10^7$ tachyzoites of Nc 1 strain. Multiple sections of central nervous system, heart, lung, liver and placenta were examined by routine histological methods. *N. caninum* characteristic lesions were evaluated according to their severity as follow: no lesion (=0), mild (=1), moderate (=2) and severe (=3) lesions. The information recorded from each experimental group were statistically analyzed observing a lower score lesion only in specimens from Group A ($p<0.05$). Either fetuses or placentas from groups receiving the experimental vaccines formulated with native antigens or recombinant proteins of *N. caninum* did not differ from those observed in specimens from Groups D and E.

Characterization of four monoclonal antibodies against *Besnoitia besnoiti* protein disulfide isomerase (PDI)

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Besnoitia besnoiti is an apicomplexan parasite responsible for bovine besnoitiosis, a high prevalence disease in tropical and subtropical regions and re-emerging in Europe. This disease is associated with great economic losses and has no effective therapy available. Protein disulfide isomerase (PDI) is an essential enzyme for the acquisition of the correct three-dimensional structure of proteins. Current evidence suggests that in *Neospora caninum* and *Toxoplasma gondii*, parasites closely related to *B. besnoiti*, PDI plays an important role in host cell invasion and may represent a promising drug target. We have previously determined the *B. besnoiti* PDI gene sequence (accession number DQ490130) and produced a recombinant *B. besnoiti* PDI (recBbPDI). Here we describe the production and characterization of four monoclonal antibodies (mAb) against recBbPDI: T8a, S4a, R60b and S16p. The four mAb recognize the full length recBbPDI as well as the native *B. besnoiti* PDI by both ELISA and western blot (WB). Using a commercial ELISA kit, all four mAb were isotyped as IgG1 with a *Kappa* light chain. Truncated versions of recBbPDI (corresponding to the domains **a**, **b**, **b'** and **a'c**) were produced and used to map mAb recognition. By WB, it was observed that mAb T8a and S4a recognize the domain **a'c**, mAb R60b recognizes domain **b'**, while mAb S16p recognizes only the full length PDI. The cross reactivity with *N. caninum* and *T. gondii* PDI was also evaluated by ELISA and WB: mAb T8a does not recognizes *N. caninum* nor *T. gondii* PDI, mAb S4a, S16p and R60b label *N. caninum* PDI in both techniques and R60b also recognizes *T. gondii* PDI by WB. This panel of mAb may represent a valuable resource for future studies concerning *B. besnoiti* PDI activity and its role in host cell invasion.

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Eimeria species parasites as novel anti-bacterial vaccine delivery vectors

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The poultry industry is one of the largest agricultural industries in the world, poultry meat and eggs being an important source of dietary protein throughout the world. >50 billion chickens are commercially reared each year and are by far the most numerous livestock animals. With increased urbanization and rapid population growth there is pressure to produce livestock more efficiently without increasing food-borne zoonosis. *Campylobacter* contamination of poultry meat is increasing and is the biggest cause of food-borne diarrhoeal zoonosis in humans throughout the world. Of particular concern is an increase in numbers of birds with bacteria not only in the intestines but also in the liver, spleen and muscle tissues indicating breach of the gut barrier and systemic infection. *Campylobacter* is considered by many to be commensal and non-pathogenic for chickens but there is increasing evidence that chickens use both innate and acquired immune mechanisms to limit and control infections. There is therefore potential to develop anti-*Campylobacter* vaccines for poultry which, if effective, could reduce *Campylobacter* load in animals and in doing so significantly reduce the transmission of this zoonotic organism into the human food chain.

Our lab has developed protocols for the expression of exogenous antigens within *Eimeria* parasites and demonstrated that *Eimeria* parasites are a good vehicle for expression of *Campylobacter* antigens. Using a *C. jejuni* antigen we have shown recombinant populations of transgenic *Eimeria tenella* induced immune responses during vaccination that reduce significantly the ability of *Campylobacter* to colonise and replicate within the intestine of chickens. In this PhD project, we plan to investigate the efficacy of several additional *Campylobacter* proteins using transgenic *Eimeria* technologies to express and deliver the antigens to chickens.

Isospora suis – maternal immunization as a strategy for immunoprophylaxis in piglets

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Isospora suis, the causative agent of neonatal porcine coccidiosis, is of big economic importance for pig production. The major symptoms are heavy diarrhea and weight loss. So far, there is no immunity-based prophylaxis. Since direct vaccination of piglets would not provide protection in the first days of life when the impact on piglet health and growth performance is strongest, two studies were conducted to evaluate the protective potential of maternal immunization against *I. suis*.

The development of specific IgG, IgA, and IgM against sporozoites and merozoites in sows, the specific antibodies (Ab) content in the colostrum and milk, the Ab uptake by the piglets, and the course of disease in experimentally infected piglets was investigated after repeated infection (5 x 20,000 oocysts) of sows 2 weeks ante partum.

In the first study transfer of specific Ab from non-naïve sows to their piglets and a correlation between higher IgA-titers in piglet sera and a better fecal consistency could be shown.

In the second study piglets from infected and non-infected sows were compared. Piglets from infected sows showed a longer prepatency and a significantly better fecal consistency. Especially for IgA there was a significant correlation between a less severe course of the disease and higher Ab-titers in the milk.

The results lead to the conclusion that ante partum infections of sows with *I. suis* have a positive influence on the clinical outcome of neonatal piglet coccidiosis. If this effect is based only on antibodies or also on the colostral transfer of specific lymphocytes still needs to be elucidated. Additionally, the identification of immunogenic and testing of application strategies developed for *Eimeria* could provide a basis for the development of a maternal vaccination strategy without the need for an infection of sows.

Immunoprophylactic efficiency against coccidiosis in broilers by transmission of maternal antibodies from CoxAbic® vaccinated hens

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Eimeria maxima is considered to be one of the most antigenic coccidian species in chickens. CoxAbic® (ABIC, Israel) is a subunit vaccine composed of purified proteins extracted from the gametocyte stage of the parasite. The result of vaccination of breeders with CoxAbic® is production of specific antibodies that are passed to their offspring through the egg-yolk.
The aim of this experiment was to check the prophylactic efficacy of the vaccine in broilers obtained from immunized parents in a field trial. In 2006, 18.000 breeders (Cobb500) were vaccinated two times, at 16 and 19 weeks of age. The cocks represented the control group (unvaccinated). The level of specific antibodies in sera was determined using a specific CoxAbic® ELISA kit, before and after vaccination monthly till 35 weeks of age. The offspring were divided in two groups of 18.000 chicks: CoxAbic group, unmedicated and control group, medicated (salinomycin). The immunoprophylactic efficiency of CoxAbic vaccine in broilers was estimated by: weight gain, food conversion, mortality and number of oocysts in the litter (OPG).
In vaccinated breeders (S/P 0.341±0.365) the level of antibodies was significantly higher than in control group (S/P 0.014±0.041) at 3 weeks after second vaccination. The broilers (OPG $8,6X10^4$) obtained from vaccinated breeders shed less oocysts at 38 days age than medicated broilers (OPG 13,8 $X10^4$). The percentage of mortality in CoxAbic chicks was 2.91% and in medicated chicks 2.8%. The weight gain was higher in medicated group (2.182 kg/chick) than in vaccinated group (1.987 kg/chick), but the feed conversion was greater in CoxAbic chickens (1.975 kg feed/kg spore) instead in control group (2.135 kg feed/kg spore).
The CoxAbic vaccine (Abic, Israel) assures an efficient passive immune protection in the descendent broilers in the lack of the use of coccidiostats.

Cryptosporidium baileyi - predictive infection model for calf cryptosporidiosis?

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Cryptosporidiosis is a world-wide protozoal parasitic infection of humans, domestic animals and wildlife. In man cryptosporidiosis can cause life-threatening diarrhea in immune-compromised individuals, children and the elderly. Testing of compounds against these zoonotic species of *Cryptosporidium* in calves is costly and requires large quantities of compound. Thus, an inexpensive animal model suitable for efficient drug evaluation *in vivo* is highly desirable. For this, the avian non-zoonotic species *Cryptosporidium baileyi* was used to establish an infection model in chicken.

In these studies, chickens were infected with *C. baileyi* oocysts and compounds were mixed into non-medicated, complete chicken feed or in drinking water. Administration lasted from day -1 to day 10 post infection (PI). For the analysis of oocysts per gram of faeces (OPG), faeces were collected on day 7 to 9 PI, and severity of infection was rated by assigning scores to the determined OPG. Furthermore, the number of oocysts in the Bursa of Fabricius was evaluated microscopically on the day of autopsy (day 10 PI) using carbolfuchsin-staining. Weight gain of treated and control groups was recorded. Histological and molecular techniques were employed to detect and quantify *C. baileyi* oocysts in host tissue samples.

To potentially reduce animal experiments in the evaluation of potential drug candidates, an *in ovo* parasite reproduction model is being explored.

Could risedronate be used in calves with cryptosporidiosis?

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Diarrhoea caused by *Cryptosporidium parvum* is a major problem in calves younger than 4 weeks of age. The aim of the present study was to emphasize prophylactic and therapeutic efficacy of risedronate, a bisphosphonate compound, as a novel approach in calves with experimentally induced *C. parvum* infection. The material of the study comprised 3-5 days old 15 healthy male Holstein calves. Calves were hospitalized in individual boxes and fed in 2 portions daily with commercially available milk replacer. All animals kept under clinical examination were monitored for fecal consistency and appetite. Each calf received $1x10^7$ *C. parvum* oocysts perorally. Calves infected with *C. parvum* were enrolled into 3 different groups (n=5). The 1st group defined as the prophlactic efficacy group received oral risedronate at a dosage of 0.5 mg/kg/day for 5 days, 1 day prior to the inoculation. The 2nd group defined as the treatment group received oral risedronate at a dosage of 0.5 mg/kg/day for 5 days, after the first day diarrhea was defined. The 3rd group defined as the control group left as placebo. Drug efficacy was assessed by evaluating severity of diarrhea and oocyst shedding from days 1 to 28. Preliminary findings indicated that there were significant differences as for the number of oocysts shed and the incidence of fecal diarrhea among the groups. Risedronate suppressed the oocyst shedding significantly and resulted in significant improvements in clinical signs. Results obtained from the present study suggest that the use of risedronate in the diet may facilitate the reduction of environmental contamination by cryptosporidial oocysts shed from infected calves.

Evaluation of the effect of trifluralin analogues on *in vitro Babesia bovis* cultures

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Babesiosis is caused by intraerythrocytic protozoan parasites of the genus *Babesia* that infect a wide range of domestic and wild animals and is highlighted as an emerging zoonosis in humans. *Babesia* sp. parasites are transmitted by ticks being prevalent worldwide, mainly in tropical and sub-tropical areas. Serious economic damage in the livestock industry has been caused by *Babesia* sp. infections in such areas. The only preventive treatment available against bovine babesiosis is based in live-attenuated vaccines, which limits its applications in several countries. In addition, there are a number of babesiacides, but only a few drugs are currently available.

Trifuralin derivatives specifically bind alpha-tubulin in plants and protozoa parasites causing growth inhibition. A set of trifuralin derivatives has previously shown to be inhibitory for the growth of *Leishmania* species. Conservation of several key amino acids involved in the trifuralin binding site of alpha-tubulin among *Leishmania* sp. and *Babesia bovis* parasites provides rationale for testing these compounds also as babesiacides. All of the trifuralin analogues compounds tested against Mo7 and Texas strains showed strong *B. bovis* growth inhibition.

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