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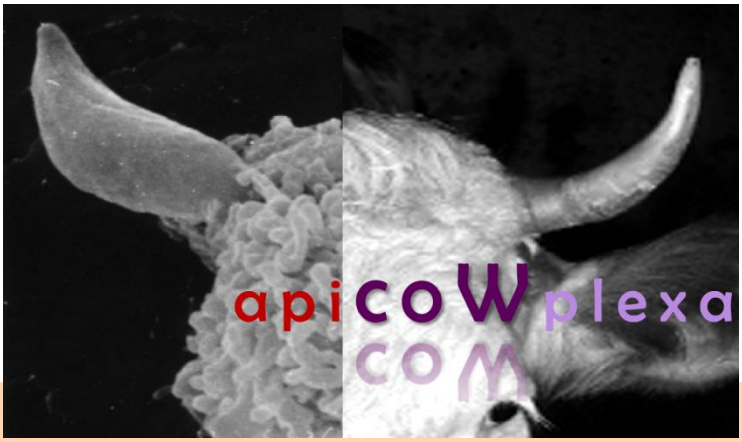

2nd International Meeting on
Apicomplexan Parasites in Farm Animals

October 31 – November 2, 2013

Kuşadası, Türkiye

ApiCOWplexa 2013

2nd International Meeting on Apicomplexan Parasites in Farm Animals

PROCEEDINGS

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Welcome

Apicowplexa 2013: 2nd International Meeting on Apicomplexan Parasites in Farm Animals

October 31 to November 2, 2013
Kuşadası

Dear colleagues,

Welcome to the 2nd International Meeting on Apicomplexan Parasites in Farm Animals, which is held at the Pine Bay Hotel in Kuşadası, Aydin.

The congress site provides a beautiful venue to meet your colleagues, and to facilitate networking and scientific exchange in this important field of research. The meeting will cover different aspects such as surveillance and diagnosis of diseases caused by apicomplexan parasites, host-parasite interactions at the cellular and immunological level, chemotherapy, vaccination, harmonisation of *in vitro* and *in vivo* models and genomics and proteomics.

We hope you will have an enjoyable meeting and look forward to welcoming you in Kuşadası!

The Organizing Committee

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	Opening lectures
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18:00 Keynote	Daniel K. Howe (Department of Veterinary Science, University of Kentucky) "Moving *Sarcocystis neurona* into the "omics" era. Finally."
18:30	**Welcome reception**

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15:15	Carlos Hermosilla “*Besnoitia besnoiti* tachyzoites trigger the release of bovine Neutrophil Extracellular Traps”

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Chair: Elisabeth Innes

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14:15 Keynote	Frank Katzer (Moredun Research Institute, Edinburgh) “Vaccination of pigs and lambs against *Toxoplasma gondii* reduces tissue cyst formation; safer meat for human consumption”
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Chair: Jonathan Wastling

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17:30 Sacha Hanig “Reporter gene strategies illuminating evolutionary conserved oocyst wall formation in *Eimeria*”

17:45 Kyoko Hayashida “Genome analysis of two genotypes of *Theileria orientalis* isolated in Japan and comparative genomics with lymphocyte-transforming *Theileria* species”

18:00 Brian Shiels “Genome wide expression profiling for identification of stage regulated transcription factors and binding motifs *in Theileria annulata*”

18:15 Arnab Pain “A tale of two algal genomes: evolution of parasitism in apicomplexans”

18:30 Leonhard Schnittger “Recent insights into the molecular phylogeny of piroplasmids”

18:45	Brian Cooke “The ‘shaveome’ of *Babesia bovis* infected-red blood cells”
19:00	Emanuel Heitlinger “The genome of *Eimeria falciformis* allows identification of conserved invasion strategies but diverged host interaction in the Coccidia (Apicomplexa)”
19:15	**Closing session** **Chairs: Tülin Karagenç & Andrew Hemphill**
20:00	**Congress dinner**

Opening lectures

Prevalence of *Theileria*/*Babesia* and *Anaplasma*/*Ehrlichia* species in small ruminants in Turkey and diagnostic sensitivity of single-PCR and RLB

Hüseyin Bilgin Bilgiç, Ahmet Hakan Ünlü, Serkan Bakırcı, Selin Hacılarlıoğlu, Onur Köse, Hasan Eren, Tülin Karagenç

University of Adnan Menderes, Faculty of Veterinary Medicine, Department of Parasitology, 09016, Isıklı/Aydın, Turkey

Ticks and tick borne diseases (TBDs) caused by *Theileria*/*Babesia* and *Anaplasma*/*Ehrlichia* species are very common in regions where distribution of host, pathogens and vectors overlap. Many of these diseases treat livestock production, and some are of public health concern. Theileriosis and babesiosis are very common in Turkey in both cattle and sheep. However, there are few studies on anaplasmosis and ehrlichiosis especially in small ruminants. The aim of the present study was to determine the prevalence of *Theileria*/*Babesia* and *Anaplasma*/*Ehrlichia* spp. in small ruminants and to compare diagnostic sensitivity of species-specific PCR and Reverse Line Blot (RLB) hybridization assay. A total of 1840 blood samples were collected between 2011-2013. All samples were screened using species-specific PCR for the presence of *Theileria ovis*, *T. lestoquardi*, *T. luwenshuni*, *T. uilenbergi*, *Theileria* sp MK, *Babesia ovis* and *Anaplasma ovis*, also with RLB for the presence of all known *Theileria*/*Babesia* and *Anaplasma*/*Ehrlichia* species in sheep and goats. According to composite PCR and RLB results 16.73%, 0.92%, 0.65%, 0.32%, 0.43%, 0.70% and 11.47% of samples were found to be infected with *T. ovis*, *T. luwenshuni*, *T. uilenbergi*, *Theileria* sp OT1, *B. ovis*, *B. crassa* and *A. ovis*, respectively. The present study reveals the presence of *T. luwenshuni* and *T. uilenbergi* in Turkey for the first time. Results also demonstrated a high prevalence of mix infections, 853 (46.35%) out of 1840 samples were infected with more than one parasite. The results obtained from the ongoing study indicated that *Theileria*/*Babesia* and *Anaplasma*/*Ehrlichia* species are commonly present in sheep and goats in Turkey. It can be concluded from these observations that RLB is capable of detecting most, but not all, of the mixed infections in some blood samples. However, the diagnostic sensitivity of single PCR was higher when compared with RLB. Therefore, the use of species-specific single PCR can be recommended to detect true prevalence of mixed infections especially in regions where intense infection rates with more than one parasite is expected.

Moving *Sarcocystis neurona* into the "omics" era. Finally.

Daniel K. Howe[1], Sriveny Dangoudoubiyam[1], Sivaranjani Namasivayam[2], Joshua Bridgers[2], Tommy Bullock[3], Jolanta Jaromcyzk[3], Ablesh Gautam[1], Michelle Yeargan[1], Ousman Mahmud[2], Katharine Korunes[2], Chris Schardl[3,4], Jessica Kissinger[2]

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2 University of Georgia, Department of Genetics and Center for Tropical and Emerging Global Diseases, Athens, GA, USA
3 University of Kentucky, UK-Advanced Genetic Technologies Center, Lexington, KY, USA
4 University of Kentucky, Department of Plant Pathology, Lexington, KY, USA

Sarcocystis neurona is the leading cause of neurologic disease in horses and an emerging pathogen of marine mammals. *S. neurona* cycles between multiple small-mammal intermediate hosts and the opossum definitive host, which restricts this parasite species to the Western Hemisphere. However, the genus *Sarcocystis* is cosmopolitan and encompasses well over 100 species that collectively infect a very broad host range including mammals, birds, reptiles, and fish. To accelerate gene discovery and enable current investigative technologies (e.g., transcriptomics, proteomics, etc.), a genome sequencing project has been conducted for *S. neurona*. The *S. neurona* genome sequence assembled into 172 scaffolds that suggest an approximate genome size of 124 Mb (i.e., ~2X the *Toxoplasma gondii* genome). Structural annotation of the genome assembly has been completed, and phylogenomic analyses are being done to compare the *S. neurona* genome with other members of the Apicomplexa. Importantly, the *S. neurona* sequences and annotations have been submitted to EuPathDB so that the information is accessible to the research community. Availability of the annotated genome has allowed us to examine transcriptional changes that occur during *S. neurona* asexual development. Extracellular merozoites and intracellular schizonts of S. neurona each have discrete functions that must be accomplished for the parasite to survive and disseminate. To explore transcriptional changes during asexual development, RNA-Seq data were generated from extracellular merozoites and 5 different time-points during intracellular schizont development. Not surprisingly, the schizont transcriptome was enriched for genes involved in nucleotide and protein synthesis, consistent with cell growth and budding of new merozoites. Information from the annotated *S. neurona* genome is being incorporated to better document differential gene expression during intracellular development. The resulting catalogue of novel and differentially-expressed transcripts will be a useful resource for identifying *S. neurona* genes involved in intracellular survival and propagation by this and other apicomplexan parasites.

Surveillance and diagnosis of apicomplexan parasites in farm animals

Bovine toxoplasmosis: fact or fiction?

Joke van der Giessen[1], Radu Blaga[2], Isabelle Villena[3], Marieke Opsteegh[1]
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Natural infection with *Toxoplasma gondii* does not appear to cause clinical disease or abortion in cattle. It has also been suggested that cattle do not readily acquire infection and, if infected, tissue cysts are unlikely to persist. Despite, the prevalence of antibodies against *T. gondii* in cattle is generally high. However, the validation of serology in cattle might be difficult since tissue cysts are only infrequently recovered from experimentally inoculated cattle and also rarely found in natural infected cattle or beef samples. Beef is thus often considered of low risk for human infection. In contrast, PCR based detection of *T. gondii* in bovine samples identified more positives compared to classical detection methods and in the Dutch cattle study it appears to be more frequent in seronegative than seropositive cattle. It was shown using a quantitative microbiological risk assessment approach (QMRA) that even with a low prevalence of 2% using PCR in cattle in the Netherlands, beef could contribute importantly to 67% of the predicted human infections depending on consumed amounts and preparation habits. One popular beef product eaten raw, filet-americain, contributed to 38% of the predicted Dutch human infections. However, PCR based detection of *T. gondii* DNA does not necessarily mean that viable parasites, indicating a risk for human infection, are present. It is thus important to elucidate whether positive PCR reflects the presence of infectious parasites. Replacing the PCR prevalence with the prevalence of viable cysts (0.96%) based on bioassay obtained after a French study in cattle, the relative attribution of beef remained high at 52.5%. This French study however, selected mostly seropositive animals for the bioassay and taking into account the conclusions of the Dutch study, this could indicate an underestimation of the true attribution. In addition, eating raw beef has been identified as a risk factor that predicts recent *T. gondii* infection in pregnant women. Consumption of raw or undercooked beef was identified as the most probable source of infection in 4 out of 26 outbreaks of human toxoplasmosis between 1965 and 2001. In conclusion, taking into account that cattle seem resistant to the clinical outcomes after *T. gondii* infection, bovine toxoplasmosis is more fiction than fact. However, the fact that recent insights using PCR and bioassay studies in cattle combined with QMRA based methods indicate that cattle play a more important role in the transmission route to humans, more insights in the relationship between seroprevalence and the presence of tissue cysts in cattle and risk factors need to be elucidated before adequate prevention measures can be implemented.

Control options for *Neospora caninum* – is there anything new or are we going backwards?

Michael P. Reichel[1, 2]*, Milton M. McAllister[1], William E. Pomroy[3], Carlos Campero[4], Luis M. Ortega-Mora[5] and John T. Ellis[2,6]

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Control options for *Neospora caninum* have in recent years become more restricted, with the withdrawal from sale of the only commercially available vaccine. While researchers continue to work on efficacious alternative vaccines, the present state of control options remains limited. Recent work has highlighted and enumerated the economic input of annual losses due to *N. caninum* abortions world-wide.

At the practical level however, recommendations for "Test-and-cull", or "not breeding from sero-positive dams" stand diametrically opposed to alternative options put forward that suggest a primary producer is better advised to keep those cows in the herd that are already sero-positive, i.e. assumed to be chronically infected, and indeed those that have already aborted once. Treatment with a coccidiostat has been put forward as the only economically viable option, yet no such treatment has gained official, regulatory approval.

The present review canvasses the relevant literature for evidence for proven control options for *N. caninum* and assesses them in the light of the authors' knowledge and experience with control of *N. caninum*.

Multiplex PCR for diagnosis of *Theileria luwenshuni*, *T. ovis* and *T. uilenbergi* in small ruminants

Xiao Zhang*, Zhijie Liu, Jifei Yang, Ze Chen, Haining He, Guiquan Guan, Aihong Liu, Qiaoyun Ren, Jianxun Luo, Hong Yin, Youquan Li

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This paper provided a multiplex polymerase chain reaction assay to simplify the species of three *Theileria* spp. complex in small ruminants. Three pairs of specific and sensitive primers were designed based on 5.8S ribosomal RNA gene (*Theileria luwenshuni*, *T. ovis*) and 28S ribosomal RNA gene (*T. uilenbergi*) to generate target products of 884 bp, 530 bp and 303 bp, respectively. This method was established with standard DNA of different species, and then a preliminary application was made among 86 field samples in China. Optimal conditions of primer concentration, annealing time and amplification cycles were tested. The sensitivity in this condition was 10-3% parasitemia and the specificity was 100%. The multiplex PCR represents a simple and efficient test method, which may be a practical alternative for rapid detection and identification of *Theileria* species of small ruminants in China.

Evaluation of two copro-antigen tests and comparison with polymerase chain reaction in the diagnosis of *Cryptosporidium* in animals and human

Yosra A. Helmy[1], Jürgen Krücken[2], Karsten Nöckler[3], Georg von Samson-Himmelstjerna[2], Karl-H. Zessin[1]

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2 Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, 14163 Berlin, Germany
3 Federal Institute for Risk Assessment (BfR), 12277 Berlin, Germany

For detection of *Cryptosporidium* species in 804 animals (593 cattle and 211 buffaloes) and 165 diarrheic children (<10 years) in Egypt, two commercial copro-antigen tests, the RIDASCREEN® Cryptosporidium Enzyme Immunoassay (EIA) and the RIDA®QUICK Cryptosporidium/Giardia combi Immunochromatographic Assay (ICT) were compared with the polymerase chain reaction (PCR) which was used as reference method. Prevalence of *Cryptosporidium* was 15.0%, 19.5% and 32.3% in animals and 2.4%, 6.7% and 49.1% in humans using the EIA, the ICT and PCR, respectively.

For animal samples, sensitivity (Se) of the EIA was 46.5% (95% CI: 40.4-52.8%) when questionable samples were considered positive whereas specificity (Sp) was 100% (95% CI: 99.3-100%). Se of the ICT was 60.4% (95% CI: 54.2-66.4%) while Sp was 100% (95% CI: 99.3-100%). The positive predictive values (PPVs) for both EIA and ICT test were 100% (95% CI: 97-100% for EIA and 97.7-100% for ICT). The negative predictive values (NPVs) for EIA were 79.7% (95% CI: 76.4- 82.6%) and 84.1% (95% CI: 81.0-86.8%) for ICT. For children samples, the Se of EIA was 5% (95% CI: 1.4-12.2%), Sp was 100% (95% CI: 95.7-100%), PPV was 100% (95% CI: 40.2 - 100%) and NPV was 52.2% (95% CI: 44.2- 60.1%) while the Se of ICT was 13.6% (95% CI: 7-23%), Sp was 100% (95% CI: 95.7-100%), PPV was 100% (95% CI: 71.3- 100%) and NPV was 54.6% (95% CI: 46.3- 62.6 %).

The Kappa score of agreement between PCR and ICT was 67.4%, 54.1% between PCR and the EIA and 84.4% between the ICT and the EIA. 9×10^3/µl of Cryptosporidia oocysts in feces were detected till the 2nd serial dilution in EIA and ICT, and until the 6th serial dilution in PCR. Results show that copro-antigen tests were easy to perform and less time consuming for *Cryptosporidium* detection but less sensitive compared to PCR.

Species and genotypes of *Cryptosporidium* found in calves from birth to one year

Sarah Thomson[1,2], Callum Harvey[1,2], Beth Wells[1], Frank Katzer[1], Nicholas Jonsson[2], Elisabeth A. Innes[1]

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2 School of Veterinary Medicine, The University of Glasgow, 464 Bearsden Road, Glasgow, G61 1QH, United Kingdom

At present there is limited knowledge of the species and subtypes of *Cryptosporidium* found in cattle on Scottish farms. In particular little is known about the shedding profiles (age and duration) of the different species found in cattle.

A longitudinal study was conducted to assess the shedding profile of *Cryptosporidium* species in a group of calves from birth to twelve months. Faecal samples were collected from 30 calves on a farm in Midlothian three times per week for the first six weeks of their lives and then again at 3, 6, 9 and 12 months. Samples were tested for *Cryptosporidium* species and genotypes by nested species specific multiplex PCR amplification and sequencing.

Four hundred and twenty-nine samples were collected throughout this study and preliminary results show that overall prevalence of *Cryptosporidium* was 60% with *Cryptosporidium* spp. being detected in 64% of samples from calves under 6 weeks and 22%, 63%, 44% and 18% from 3, 6, 9 and 12 month old calves respectively. Three of the common cattle species of *Cryptosporidium* were detected throughout the study with most calves under 6 weeks being predominantly infected with/shedding *Cryptosporidium parvum*, in the other age groups most calves were infected with/shedding *Cryptosporidium bovis* or *Cryptosporidium ryanae* except at six months where, unusually, most animals were infected with/shedding *C. parvum*. Mixed infections of these species were also detected in calves <6 weeks old and >6 months old. GP60 genotyping of selected *C. parvum* positive samples from calves <6 weeks all show genotype IIaA19G2R1 whereas *C. parvum* positive samples from older animals are all IIaA15G2R1, further genotyping and microsatellite analysis is ongoing.

Blood content in meat juice samples influences the detection of antibodies to *Toxoplasma gondii*

Camilla Gustafsson[1]*, Arvid Uggla[1], Ivar Vågsholm[1], Jenny Frössling[2,3], Alison Burrells [5], Anna Lundén[1,4]

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The use of meat juice for serological screening of *Toxoplasma gondii* infections in animals has many advantages. However, serological assays based on meat juice instead of serum might have inferior sensitivity (1), and it has been suggested that this could be overcome by adjusting for blood content in the meat juice (2). In the present study, blood content, measured as haemoglobin and/or total IgG concentration, in meat juice samples from different muscles, and its effects on the apparent level of *T. gondii* specific antibodies, were studied.

Serum and six different muscle samples (diaphragm, tongue, heart, Musculus semitendinosus, M. triceps, M. longisimus dorsi) were collected from 20 pigs inoculated with either *T. gondii* oocysts, tissue cysts or tachyzoites six weeks prior to slaughter, and from three uninfected controls. Meat juice was extracted by freezing and thawing tissue samples. The concentration of haemoglobin was calculated by subtracting the myoglobin concentration, measured by a commercial ELISA, from the total heme pigment concentration measured by the alkaline haematin detergent method (AHD-575) (3). Total IgG levels were measured by capture ELISA and *T. gondii* specific antibodies were detected by an in-house ELISA.

Significant differences in haemoglobin concentration ($p < 0.001$) and total IgG concentration ($p < 0.05$) between meat juice samples from different muscle types were found, and for diaphragm samples there was a significant correlation between haemoglobin and total IgG content ($p < 0.05$). Furthermore, preliminary results indicate that the level of *T. gondii* specific antibodies is dependent on the total IgG concentration and the total pigment concentration in the meat juice sample.

These results highlight the need to define the sampling and preparation procedure for meat juice samples to be used in serological assays since variations in blood content will affect test performance.

References: 1-Gamble et al. Veterinary Parasitology 2005, 128: 177-181; 2-Mecca et al. Meat Science 2011, 88: 584-589; 3-Zander et al. Clinica Chimica Acta 1984, 136: 83-93.

Distribution of *T. gondii* tissue stages after experimental infections of broiler chickens

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Toxoplasma (*T.*) *gondii* is a widely spread protozoon in humans, mammals and poultry forming tissue cysts in several organs especially in neural and muscular tissue. More and more poultry is kept in free-range husbandry which renders the risk for an infection with *T. gondii* high.

Therefore, we simulated the natural ways of infection and investigated the distribution of *T. gondii* tissue cysts in different organs of experimentally infected chickens.

48 one-week-old chickens were infected orally with $1x10^6$, $1x10^5$, $1x10^3$ oocysts or tissue cysts in mouse brains (n=6) of a *T. gondii* field strain (Type II) from the Czech Republic or ME49 strain, respectively. 12 animals were kept as uninfected control groups. Weekly, blood was drawn to determine the serotiter. All chickens were slaughtered 5 weeks after infection. Organ samples from 16 different localizations were analysed for *T. gondii* with a nested PCR based on the B1 gene.

All infected chickens were tested seropositive for *T. gondii*-antibodies by IFAT and p30 immunoblot.

Regarding field strain infections, *T. gondii* DNA was detected in 65 of 384 total organ samples (16.9%) and in 27 samples of edible organs (7.0%), respectively. 21 of 24 chickens were tested positive for *T. gondii*-DNA in at least one organ. There was no significant difference between the infection groups regarding the number of positive tissue samples or the type of tissues parasitized. Results of ME49 infections are pending.

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Risk factors for *Toxoplasma gondii* infections in pigs in Germany

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The aim of the study was to estimate the prevalence of antibodies to *Toxoplasma gondii* and to identify potential risk factors for infection in domestic pigs in Lower Saxony, Germany. Porcine plasma samples collected in monitoring programs for Aujeszky´s Disease and Classical Swine Fever were examined in a modified commercial *T. gondii* ELISA. To determine risk factors for seropositivity to *T. gondii* in pig rearing units, farmers were interviewed by telephone using a standard questionnaire. For 260 units examined by ELISA, questionnaire data were obtained. A unit was regarded as seropositive if at least one animal had tested positive for antibodies to *T. gondii*. Antibodies to *T. gondii* were detected in a total of 51 units (19.6%). The lowest prevalence was observed in fattening pigs (6.9%) and the highest in sows (31.2%). To identify potential risk factors for seropositivity to *T. gondii*, the data were first analyzed by univariate logistic regression, which revealed many variables statistically significantly associated with seropositivity. They included factors linked to the presence of cats, feeding practices, storage of fodder, cleaning and hygienic measures. A multivariate logistic regression model showed that presence of cats in stables, storage of fodder in open containers and manual feeding were statistically significant risk factors and revealed a >4 days service period until re-stabling as a significant protective factor.

The study was funded by BMBF (01 KI 0765, Toxonet).

Algerian local cattle were naturally resistant to abortion caused by *Neospora caninum*

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Neospora caninum is an Apicomplexa parasite causing abortion in cattle worldwide. The situation in Algerian cattle was unknown. 799 cattle belonging to 87 farms of the north and north east of Algeria were investigated. First a seroprevalence study was performed. The global seroprevalence in IFAT was 19.64%. The cattle were divided into modern cattle corresponding to imported cattle, local cattle corresponding to local breeds and improved cattle corresponding to breeding between modern and local cattle. The seroprevalence was 16.04%, 18.64 % and 34.28% in modern cattle, improved cattle and local cattle, respectively.

The study of risk factors indicated that the race, the geographical area, the presence of dogs, the seasons, the global hygiene of the farm and the presence of abortion were positive risk factors for the prevalence of *N. caninum*.

A case control study has been performed to investigate the link between the seropositivity to *N. caninum* and the abortion in cattle farms. There is a clear significant ($p < 0.01$) association between the seroprevalence against *N. caninum* and the presence of abortion in farms (OR = 12.03). These association was also significant at the individuals levels (OR = 2.79).

For the several cattle populations, the association is more contrasted, there is a clear association between seroprevalence and abortion in modern ($OR=\infty$) and improved (OR = 9.62) cattle but the association was not significant for local cattle (OR = 1) even if it was the population where the seroprevalence was the highest. Therefore, the local Algerian cattle races were infected by *N. caninum* but they seem to resist to abortion.

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NOT PRESENTED

A molecular study of congenital neosporosis associated with abortion in sheep in Mashhad area, Iran

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Neosporosis is an important cause of abortion and stillbirth in dairy cattle in the worldwide. Ovine abortion due to *N. caninum* infection has been reported in some countries. In this study, we tried to determine the role of *N. caninum* in inducing abortion in sheep of Mashhad area, the Khorasan Razavi Province, Iran. Seventy one brains of aborted ovine fetuses were collected during the lambing season between 2010 and 2012. DNA of brain samples were extracted and examined for the presence of *Neospora* using PCR Method. *N. caninum* was detected in the brain samples of seven (9.8%) of aborted fetuses. The results indicated that neosporosis could be a causative agent of abortion in sheep and need more investigation.

Follow-up study of a *Neospora caninum* exogenous transplacental transmission episode in a high producing dairy herd

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Neospora caninum exogenous transplacental transmission occurs as a consequence of a primo-infection of pregnant cattle after ingestion of oocysts shed by a dog (horizontal transmission) and it is frequently associated with abortion outbreaks. Currently, the importance of this transmission route on the persistence of infection in herds and on the reproductive outcome in subsequent pregnancies is controversial. The aim of this study was to investigate the infection dynamics in a high-producing dairy farm, following an outbreak of abortion cases associated with *N. caninum* infection and without a previous history of neosporosis. Thus, serological and reproductive parameters were evaluated in the herd composed of 1000 animals on average with 650 lactating cows. After the abortion outbreak, blood samples of all animals older than 6 months were collected in three different samplings during a period of 1 year and tested for anti-*N. caninum* antibodies. Data on birth dates, abortion and dam-daughter pairs were annotated for every animal and obtained from the management program operating in the herd. The results support the hypothesis that postnatal infection could be transient in some animals and that postnatally infected cattle could less efficiently transmit the infection to progeny than congenitally infected animals based on the following results: i) a significantly decrease of seroprevalence throughout the study (from 16.9% to 12%; $P<0.005$, $\chi 2$) was observed; ii) low antibody levels were detected in most of the seropositive animals that fluctuated around the cut-off, and 70 out of 161 seropositive animals turned into seronegative; iii) the proportion of aborting and seropositive dams decreased from 81.8% (abortion outbreak) to 12.9% (during the study period) and no association between seropositivity and abortion was found; and iv) the transplacental transmission rate of seropositive dams to offspring that were born throughout the study period was 28.57%.

Eimeria infection in dairy cattle of industrial farms in Kangavar suburban of Kermanshah province, Iran

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Eimeria infection is of importance protozoal infection in ruminants which causes economic losses in animal husbandry of Iran and worldwide. This study was aimed to determine prevalence and *Eimeria* species diversity in dairy cattle with different age groups under industrial management. The present study was carried out in industrial dairy cattle husbandry of Kangavar suburban of Kermanshah province, Iran, from spring 2011 to 2012. A total of 307 fresh fecal samples were randomly collected and subjected for flotation technique. The intensity of infection was determined using McMater method. The overall prevalence was 31.92% which a total of 98 out of 307 fecal samples were positive. The highest prevalence was found in dairy cattle (37%) with 1-3 years old. There was no significant difference between prevalence and different age groups. The highest frequency was significantly in spring (41.66%). The intensity was ranged from 3.18×10^3 to 2.71×10^5. There was no significant difference between the prevalence and intensity in all age groups. Fecal consistency findings revealed that the highest infection was significantly in dairy cattle with normal fecal consistency in all age groups. According to Laboratory identification, a number of seven *Eimeria* species were detected in all infected animals. The most common specie was *E. zuernii* (28.25%) in 5% dairy cattle (1-3 years-old) in spring (30%) and summer (30%). There was significant correlation between frequency of *Eimeria* infection and season. All infected cattle had mixed infections with two (42.85%) and three (3.15%) species of *Eimeria*. The results of this study elucidated cattle *Eimeria* infection is a problem in dairy cattle of industrial farms in the region. Attention should be paid within seasonal infection in young dairy cattle to avoid clinical coccidiosis, particularly in farms with poor hygienic conditions and no prophylactic treatments.

Host-parasite interactions at the cellular and immunological level

Telomerically encoded determinants of *Cryptosporidium* virulence

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We sought to identify species specific genes that might dictate host specificity and serve as gold standards for species determination and markers of virulent strains. We identified a family of telomerically encoded genes, two of which display overt heterogeneity between species. Although initial work suggested both were species specific, Cops-1 for *C. parvum* and Chos-1 for *C. hominis*, subsequent study identified an abridged ortholog of Cops-1 in *C. hominis*. Cops-1 and Chos-1 showed limited, but significant, similarity to each other and share common features: (i) telomeric location: Cops-1 is the last gene on chromosome 2, whilst Chos-1 is the first gene on chromosome 5, (ii) encode circa 50-kDa secreted proteins with isoelectric points above 10, (iii) are serine rich, and (iv) contain internal nucleotide repeats. Importantly, those members of the gene family with orthologs in both species show sequence variation with good discriminatory power useful epidemiologically. *C. parvum*-infected patient sera recognized a 50-kDa protein in antigen preparations of *C. parvum* but not *C. hominis*, consistent with Cops-1 being antigenic for patients. Interestingly, anti-Cops-1 monoclonal antibody (9E1) stained oocyst content and sporozoite surface of *C. parvum* only. This study provides a new example of protozoan telomeres as rapidly evolving contingency loci encoding putative virulence factors. We have discovered a family of telomerically encoded secreted glycoproteins which show considerable variation in sequence between *C. parvum* and *C. hominis.* In particular at least two of these genes appear to lack syntenic orthologs in their sister genomes making them good candidates for strain and species specific molecular probes.

The level of H_2O_2–type oxidative stress regulates virulence of *Theileria*-transformed leukocytes

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Theileria annulata infects predominantly macrophages, and to a lesser extent B cells, and causes a widespread disease of cattle called tropical theileriosis. Disease-causing infected macrophages are aggressively invasive, but this virulence trait can be attenuated by long-term culture. Attenuated macrophages are used as live vaccines against tropical theileriosis and via their characterisation one gains insights into what host cell trait is altered concomitant with loss of virulence. We established that sporozoite infection of monocytes rapidly induces *hif1*-α transcription and that constitutive induction of HIF-1α in transformed leukocytes is parasite-dependent. In both infected macrophages and B cells induction of HIF-1α activates transcription of its target genes that drive host cells to perform Warburg-like glycolysis. We propose that *Theileria*-infected leukocytes maintain a HIF-1α-driven transcriptional programme typical of Warburg glycolysis in order to reduce as much as possible host cell H_2O_2-type oxidative stress. However, in attenuated macrophages H_2O_2 production increases and HIF-1α levels consequently remained high, even though adhesion and aggressive invasiveness diminished. This indicates that *Theileria* infection generates a host leukocyte hypoxic response that if not properly controlled leads to loss of virulence.

Eimeria bovis modulates endothelial host cell cholesterol metabolism for successful replication

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During first merogony *Eimeria bovis* forms large macromeronts of up to 400 µm size containing >120,000 merozoites. As the sporozoite stage cannot provide all components necessary for this nutrient and energy demanding process, the parasite needs to scavenge molecules from the endothelial host cell. Especially for the offspring's membrane production, large amounts of cholesterol are indispensable for a successful replication process. We here analysed the parasite's influence on cholesterol and lipid contents of the host cell and examined several molecules being involved in both host cell derived *de novo* synthesis of cholesterol and receptor-mediated uptake of low densitiy lipoproteins (LDL) from the extracellular compartment.

Kinetic analyses on developing *E. bovis* meronts I revealed esterified cholesterol and fatty acid accumulation in form of lipid body deposition in infected host cells as detected by Nile Red staining. Artificial increase of cellular lipid bodies via oleic acid treatment led to significantly increased merozoite production. Furthermore, cholesterol tracer (filipin) showed strong fluorescent signals in infected bovine endothelial cells. Cholesterol quantification assays confirmed increased cholesterol contents in infected monolayers. Several gene transcripts of molecules being involved in cellular cholesterol *de novo* synthesis via the mevalonate biosynthesis pathway (e. g. HMG-CoA synthase, HMG-CoA-reductase and squalen synthase) were found up-regulated towards the end of macromeront formation. Simultaneously, gene transcripts of the LDL-receptor and the oxidised LDL receptor 1 were induced during *E. bovis* infection. Furthermore, cellular cholesterol processing appeared enhanced as key molecules, such as cholesterol-25-hydroxylase and acetyl-CoA acetyltransferase (ACAT) were significantly up-regulated in infected cells. Inhibition assays revealed a key role of ACAT since ACAT inhibition completely blocked *E. bovis* development. In contrast, blockage of squalen synthase led to less prominent effects.

Overall, these results indicate that *E. bovis* massively exploits the host cell cholesterol metabolism to guarantee its intracellular growth and replication.

Besnoitia besnoiti tachyzoites trigger the release of bovine Neutrophil Extracellular Traps

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Bovine besnoitiosis is an important emerging protozoan disease in Europe causing economic losses and severe clinical signs in affected animals. Neutrophil extracellular trap (NET) formation was recently demonstrated as an important innate effector mechanism of polymorphonuclear neutrophils (PMN) acting against several invading pathogens. We here studied interactions of bovine PMN with tachyzoites of *B. besnoiti* with respect to NETosis. Fluorescence analyses as well as scanning microscopy analyses showed NET formation rapidly being induced upon contact with *B. besnoiti* tachyzoites. Co-localization of extracellular DNA with histones (H3), neutrophil elastase (NE) and myeloperoxidase (MPO) in parasite entrapping structures confirmed the classical characteristics of NETs. In contrast to MPO, tachyzoite-triggered NE enzymatic activity as well as ROS production were significantly enhanced in tachyzoite-exposed PMN. Consequently, inhibition of NE and NADPH oxidase led to significant diminishment of NET release, whilst blockage of MPO did not influence NETosis. In addition, NETs were also abolished by DNase treatment. Overall, these results indicate a key role of ROS and NE, but not of MPO in *B. besnoiti*-triggered NETosis. Furthermore, NETosis occurred irrespective of parasite viability since vital and dead tachyzoites equally induced NET formation. However, the use of crushed parasites promoted significantly less NETs when compared to intact ones, indicating a certain role of parasite integrity. Tachyzoite entrapment assays revealed a proportion of 28% to be immobilized in NET structures. In consequence, tachyzoites were hampered from active invasion of host cells. Thus, the transfer of tachyzoites, previously being confronted with PMN, to adequate host cells resulted in significantly reduced infection rates (40%) when compared to PMN-free controls.
We here report for the first time on *B. besnoiti*-induced NET formation. Our results indicate that NETosis may represent an important effector mechanism of the host early innate immune response against *B. besnoiti*.

Characterization of the immune cell response in the placentas from cattle following experimental inoculation with *Neospora caninum* throughout pregnancy

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Despite *Neospora caninum* (NC) being a major cause of bovine abortion worldwide, its pathogenesis is not completely understood. Evidence of immune mediated placental pathology has been reported as being responsible for compromising pregnancy probably due to the adverse effect of an exacerbated Th1 response at the maternal-foetal interface. Different clinical outcomes are known to follow experimental infections at different stages of gestation, with foetal death being the most common finding during early gestation infections, and the birth of live congenitally infected calves upon infection at mid or late gestation. The aim of our studies was to characterise placental immune responses following experimental infection during pregnancy. Cows were infected with NC tachyzoites at day 70, 140 and 210 of pregnancy and culled at 14, 28, 42 and 56 days post inoculation. Placentomes were examined by immunohistochemistry using antibodies against macrophages, T-cells (CD3, CD4, CD8, γδTCR), NK and B cells and by *in situ* hybridization to characterize cytokine expression (IL-12, IFN-γ, TNF-α and IL-4). Inflammation was mainly characterised by the presence of $CD3^+$, $CD4^+$ and γδ T-cells during the three time points. In early gestation inflammation was generally moderate to severe and mainly characterized by infiltration of IL-12, IFN-γ and TNF-α expressing cells. This infiltration was more pronounced in the samples of placentome collected from dams carrying a dead foetus or one that had aborted, compared with the mothers carrying live foetuses at the time of sampling. In contrast, the infiltration of $CD3^+$, $CD4^+$, $CD8^+$ and γδ T-cells and Th1 cytokine expressing-cells was less evident following NC infection at mid gestation and scarce during infection at late gestation. These findings may partially explain the milder clinical outcome observed when animals are infected with NC at mid or late gestation.

The interaction of microtubule plus end tracking proteins (+TIPs) with the *Theileria annulata* schizont

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The strictly intracellular *Theileria* schizont 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 (MTs), is crucial for the faithful segregation of the parasite between two daughter cells. More recently we reported that the microtubule plus end tracking protein (+TIP) EB1, the core component of +TIP networks and an important regulator of microtubule dynamics, is recruited to the parasite surface in a cell-cycle dependent manner via the membrane protein p104. Here we expand upon our investigations into *Theileria* – MT interactions, and describe the striking interaction of the MT-stabilizing +TIP CLASP1 with the schizont surface. The interaction of CLASP1 with the parasite surface does not require MTs, and unlike EB1, is cell cycle independent.

Toxoplasma gondii MyoH: an essential motor implicated in the glideosome functions

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The phylum of Apicomplexa groups protozoan parasites infecting humans and animals. Host cell invasion and egress from infected cells are key events in the lytic cycle of these obligate intracellular pathogens. Host cell entry is powered by gliding motility and initiated by the attachment and reorientation of the parasite such that micronemes and rhoptries sequentially discharge their contents at the site of contact with the host cell. The glideosome is the molecular machine anchored at the parasite pellicle and composed of myosin A as well as a number of associated proteins and has been implicated in motility, invasion and egress. The coccidian-subgroup of Apicomplexa possesses a motile organelle called the conoid, which is positioned at the extreme apical pole of the zoite and consists in a unique polymer of tubulin fibers. The conoid protrudes during invasion and egress in a cytochalasin D sensitive manner that suggests the implication of the actomyosin system. The myosin H (TgMyoH) localises to the apical ring of the conoid. This class XIV myosin exhibits 6 to 8 IQ motifs and an alpha tubulin suppressor (ATS) domain. Conditional disruption of TgMyoH established the essential nature of this motor for parasite survival. Detailed phenotypic analysis of the mutant revealed that TgMyoH is critical for gliding, invasion and egress. Neither micronemes nor rhoptries secretion are affected by the depletion of TgMyoH, and these parasites are still capable of protruding their conoid in response to calcium ionophore treatment. Two myosin light chain (MLC5 and MLC7) have been identified as partners of TgMyoH, probably involved in the regulation and/or trafficking of the myosin. Moreover, an interaction between TgMyoH and microtubules has been established *in vitro* and could be linked to the observed phenotypes involving the glideosome functions. Identification of potential cargo/partners of TgMyoH is still under investigation.

mRNA transcripts in the small intestine of *Isospora suis* - infected piglets

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Isospora suis, the causative agent of neonatal porcine coccidiosis, infects the enterocytes of the small intestine. The resulting non-haemorrhagic diarrhoea leads to reduced weaning weights and considerable economic losses in pig breeding industries worldwide. Little is known about the local reaction in the intestinal mucosa to this parasite. To further investigate mRNA expression of different genes associated with immunopathology and tissue integrity, RT-qPCR analysis of pattern recognition receptors (TLR-2, TLR-4, TLR-9, NOD2), inflammatory and immune-regulatory cytokines (TNF-α and TGF-β1, respectively) and the cytoskeleton (β-actin, vimentin, moesin) was performed. *I. suis*-infected piglets (infection with 1000 oocysts on the third day of life) were compared to non-infected control animals. Snap frozen mid-jejunum of 51 animals of 5 age groups (day of life 7, 9, 12, 15 and 18, respectively) were examined. In infected piglets the TLR-2 mRNA levels were significantly increased on day 7 (3.1-fold; $p = 0.016$) and day 15 (3.6-fold; $p = 0.008$) compared to non-infected animals. Also significantly higher NOD2 mRNA levels in infected animals were found on day 7 (3.7-fold; $p = 0.032$), day 12 (2.1-fold; $p = 0.032$) and day 15 (2.4-fold; $p = 0.016$). Infection with *I. suis* led to a significant increase of TNF-α mRNA level on day 7 (2.8-fold; $p = 0.032$). No differences between infected and non-infected piglets were found for mRNA expression levels of TLR-4, TLR-9 and TGF-β1, as well as transcripts of cytoskeleton genes. Our results indicate that TLR-2 and NOD2 are involved in the etiopathology of an *I. suis* infection and might be responsible for parasite recognition. Additionally, the simultaneous increase of TLR-2, NOD2 and TNF-α mRNA levels might suggest an interaction between these factors.

Eimeria falciformis infection of the mouse caecum identifies opposing roles of IFNγ-regulated host pathways for the parasite development

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Intracellular parasites reprogram host functions for their survival and reproduction. Conversely, the infected host attempts to defend the microbial insult. The extent and relevance of parasite-mediated host responses *in vivo* remains poorly studied, however. We utilized *Eimeria falciformis*, an obligate intracellular parasite completing its entire life cycle in the mouse intestinal epithelium, to identify and validate host determinants of the parasite infection. The most prominent mouse genes induced during the onset of asexual (24 hrs) and sexual (144 hrs) growth of parasite comprise IFNγ-regulated factors, e.g., immunity-related GTPases (IRGA6/B6/D/M2/M3), guanylate-binding proteins (GBP2/3/5/6/8), chemokines (CxCL9-11) and several enzymes of the kynurenine pathway including indoleamine 2,3-dioxygenase 1 (IDO1). These results indicated a multifarious innate defense (tryptophan catabolism, IRG, GBP, chemokine signaling) mounted by epithelial cells, and a consequential adaptive immune response (chemokine-cytokine signaling, lymphocyte recruitment). The inflammation- and immunity-associated transcripts were increased during the course of infection, following influx of B-cells, T-cells and macrophages to the parasitized tissue. Consistently, parasite growth was enhanced in animals inhibited for CxCr3, a major chemokine receptor on immune cells. Interestingly, despite a prominent induction, mouse IRGB6 failed to bind and disrupt the parasitophorous vacuole in parasite cultures, implying an immune evasion by *E. falciformis*. Surprisingly, oocyst output was impaired in IFNγ-R-/- and IDO1-/- mice, which suggests a subversion of IFNγ-signaling by the parasite to promote its growth. Collectively, our study in the *Eimeria*-mouse model identifies a retinue of host determinants regulated by IFNγ, some of which are protective, while others are subverted or even exploited by the parasite.

Tools and targets for chemotherapy

Dual use therapeutics for Cryptosporidiosis, Toxoplasmosis, and Neosporosis

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New drugs are urgently needed for cryptosporidiosis and toxoplasmosis for human and domestic livestock use. Cryptosporidiosis causes wasting diarrhea in humans and calves. Toxoplasmosis is an important cause of fetal malformations and abortions in humans and domestic livestock. In addition, an effective drug is also needed for veterinary use for neosporosis, the cause of epidemic abortions in livestock. We have created potent and selective inhibitors targeting protozoan Calcium Dependent Protein Kinases (CDPKs) of *Cryptosporidium parvum*, *Toxoplasma gondii* and *Neospora caninum*. We have two promising pre-clinical drug candidates, with different core scaffolds, that show anti-proliferative activity against these 3 parasites *in vitro*. *In vitro* screens of off target liabilities (mammalian protein kinases, G-protein receptors, and ion channels) show high specificity towards Cp/Tg/Nc CDPKs. One of our leads shows efficacy greatly reducing infection in mouse challenge models of cryptosporidiosis, toxoplasmosis and neosporosis. The second has not been tested for *in vivo* efficacy as much as the first compound, but already shows efficacy in the rodent model of toxoplasmosis. These preclinical compounds have pharmacokinetic properties consistent with once- or twice-daily dosing in rodents and show no toxicity in 5 day super-physiological dosing of rodents. Further work will include target animal pharmacokinetics, challenge studies for efficacy, assays for compound residuals and metabolism, and completion of the safety package. Thus, these compounds are of great potential value for both veterinary and human health.

Prevention of necrotic enteritis in chickens by toltrazuril

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The efficacy of toltrazuril treatment for prevention of coccidiosis and necrotic enteritis (NE) was tested. Ninety-six 14-day-old commercial broiler chickens were caged and divided into 8 groups (n=12), designated groups 1-8. Chickens of groups 1-6 were inoculated orally at 18 days of age with 25,000 oocysts of *Eimeria* (*E.*) *tenella* and 75,000 oocysts of *E. brunetti*. At 22 days of age, chickens were infected with 10^9 cfu *Clostridium* (*C.*) *perfringens*. Chickens of group 1 were treated with 75 ppm toltrazuril in drinking water for 8 h on two consecutive days up to 12 h before *Eimeria* spp. infection. Chickens of groups 2-5 were treated with the same dose of toltrazuril at 12 h, 36 h, 60 h and 84 h after *Eimeri*a spp. infection, respectively. The non-treated group 6 served as positive control. Chickens in group 7 were treated with toltrazuril at 17 and 18 days of age to assess direct effects of the compound and those of group 8 remained uninfected and non-treated as negative control. The feed conversion ratio (FCR) was higher in positive control compared to other groups. The mortality rates were 16.8% and 42% in the late toltrazuril-treated (at 84 h) and infected non-treated chickens, respectively. NE and coccidiosis specific lesion scores in infected, non-treated chickens were significantly more severe compared with negative controls ($P < 0.01$) and all infected-treated groups ($P < 0.05$). In conclusion, application of toltrazuril before *Eimeria* spp. challenge protected chickens from coccidiosis and indirectly from successive NE caused by *C. perfringens* infection.

Effect of sainfoin on lamb coccidiosis

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Weaned lambs were allocated into two treatment groups receiving diet based on either lucerne or sainfoin. The trial was performed in triplicate, and lambs were challenged with oocysts of *E. crandallis* in addition to the natural infection during the last two trials. Opg counts, faecal scores and weight gain were recorded during the trials, which lasted for 7-8 weeks. Additionally, a feeding trial which included 31 pregnant ewes and all the lambs was carried out including: group A (15 ewes, receiving sainfoin-hay) and group B (16 ewes, receiving lucerne-hay). Those ewes received the respective diet 1 month before and 3 months after lambing. The sampling period for their lambs lasted 9 weeks starting at day 9 of age.

In two of the lamb experiments (trial 1 and 3) a reduction in the mean oocyst excretion rates was observed, 3-4 weeks after sainfoin hay administration. This reduction, ranged between 21.3% (trial 1) and 61.7% (trial 3) compared to the control values. As a result a decrease in the total number of oocysts excreted was observed from week 3 or 4 to the end of those two trials respectively (reduction 48.2%, p=0.05 trial 1; 49.7% p=0.06 trial 3). When ewes were fed sainfoin hay the total amount of oocysts excreted by their lambs throughout the trial was reduced 48.1% in comparison to the control (p=0.01).

The results suggest that the use of bioactive forages, such for example sainfoin, has a potential to be used as prophylactic agents against coccidiosis in lambs but further research is needed in order to define the mechanisms involved and the treatment protocols which should be applied.

In vitro effects of novel ruthenium complexes in *Neospora caninum* and *Toxoplasma gondii* tachyzoites

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A panel of 16 compounds with anti-proliferative properties in cancer cells were investigated in *Toxoplasma gondii* and *Neospora caninum* infected human foreskin fibroblasts (HFF). The panel included 8 ruthenium complexes, several pyridine-based CYP inhibitors, and a lipophilic imidazolium salt, 1,3-bis(2,4,6-trimethylphenyl)imidazolium chloride. Only three compounds (16-18) belonging to the group of hydrolytically stable ruthenium complexes exhibited varying degrees of activity against both parasites, with compounds 16 and 18 showing IC_{50} values in *N. caninum* of 6.7 and 11.3 nM and in *T. gondii* of 18.7 and 41.1 nM, respectively. IC50 values in HFF monolayers were 2.4 and 6.95 µM, respectively. Compound 16 and 18 both severely affected the viability and infectivity of extracellular *N. caninum* tachyzoites, while in *T. gondii* tachyzoites this was observed only for compound 16. Treatments with both drugs for 3 days at concentrations up to 500 nM did not exert parasiticidal activity *in vitro*. *T. gondii* and *N. caninum* tachyzoites could both adapt to increasing concentrations of compound 18 during long term treatments (up to 45 days with step-wise increase of drug pressure). However, treatments with compound 16 at 160 nM after 27 days led to complete loss of viable *T. gondii* and *N. caninum* tachyzoites were non-viable after 22 days and at a concentration of 80 nM. TEM confirmed that compound 16 had a rapid impact and induced morphological and ultrastructural alterations in both parasites. Ruthenium-based compounds could thus represent an interesting option that should be for further pursued in studies involving animal experimentation.

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Structure-activity relationships beyond the gatekeeper residue: homologous calcium dependent protein kinases as drug targets against the Apicomplexa

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Apicomplexan parasites including *Plasmodium falciparum*, *Toxoplasma gondii*, *Cryptosporidium parvum*, *Babesia bovis*, *Neospora caninum* and *Eimeria tenella*, are obligate intracellular pathogens that must invade a host cell. Recent advances in kinase biology have shown that transmission of calcium signals under the control of calcium dependent protein kinases (CDPKs) are essential for host cell invasion. Specific inhibition of CDPKs can be achieved with Bumped Kinase Inhibitors (BKIs) based on the size of the gatekeeper residues.

Furthering previous work on apicomplexan CDPKs, we have expressed and purified homologues of veterinary pathogens *E. tenella*, *N. caninum* and *B. bovis* CDPK. A comparative analysis of the structural activity relationship of *E. tenella* and *B. bovis* CDPK against those of *P. falciparum*, *C. parvum* and *T. gondii* CDPKs was determined by screening against 349 BKIs. Based on structural models and experimental data, it was observed some residues beside the gatekeeper can influence compound-protein interactions resulting in distinct structure-activity relationships (SARs) for BKI inhibitors against individual CDPK. We have defined potential structural influence of amino acid layouts within the ATP binding cavity for individual CDPK homolog necessary for consideration in the development of broad spectrum apicomplexan CDPK inhibitors. In short, *E. tenella* CDPK1 was more resistant to the BKI library than *B. bovis* CDPK4 or *P. falciparum* CDPK1 with similar threonine gatekeeper residues because of differences in the sizes of hydrophobic pockets, charges and hydrophobicity of amino acids within the ATP binding cavities.

Vaccines

Strategies for anti-coccidial immunoprophylaxis

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Coccidiosis is a major pathogenic disease of poultry caused by parasitic protozoa belonging to the genus *Eimeria.* It is responsible for significant economic losses worldwide. Current means of control rely primarily on the prophylactic use of anticoccidial drugs that are included in the feed. Whilst cost effective to administer, the development of resistance to these drugs and public demand for residue free meat has spurred the search for alternative means of control. It is well documented that chickens that recover from infection with *Eimeria* develop immunity to reinfection. This immunity, although complex and not entirely understood, indicates a potential for immunological control and has led to the development of a number of vaccines utilising deliberate infection of chickens. These have included the use of controlled doses of virulent oocysts and reproductively attenuated lines of *Eimeria* that do not complete the usual life cycle and are, thus, less pathogenic; arguably, the most efficacious of these is Paracox®, a formulation including attenuated lines of the seven species of *Eimeria* that infect chickens. It is successful because natural immunity is primarily induced by and active against relatively early asexual stages of *Eimeria.* However, experimental vaccines with individual antigens from asexual stages of development have had only limited success. An alternative strategy for the control of coccidiosis is one based upon maternal immunity. Hens are able to transfer large quantities of protective antibodies to hatchlings via the egg yolk, and maternal infection with *Eimeria* is known to induce this phenomenon. Analysis of yolks and sera of progeny from hens infected with *Eimeria maxima* identified a relatively small number of antigenic parasite proteins. Maternal delivery of these antigens, including sexual stage proteins, is potentially economically feasible and can also confer immediate protection to newly hatched chicks, benefits not readily achieved by other immunoprophylactic control methods.

Theileria vaccines: current status and future prospects

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Theileriosis caused by infection with *T. parva* or *T. annulata* has largely been controlled by the use of acaricides to reduce tick burdens or by live vaccination against disease using either attenuated cell lines or infection and treatment (infection with a titred dose of sporozoites and tetracycline). This paper will review and discuss the efficacy of such vaccines in relation to field & experimental challenge, their use in different epidemiological scenarios, the cost/benefit of vaccination and their pro's and cons. Based on the disadvantages of live vaccines, research on the development of recombinant sub-unit vaccines has been undertaken over the last three decades. While such vaccines have not yet been developed, the progress and prospects for the future will be discussed in relation to antigen identification, vaccine trials, the type of vaccine required, and the issues of host and parasite diversity. The restraints on taking this research forward will be considered as well as the possibilities raised by recent developments for the analysis of vaccine responses and host and parasite diversity.

The rhoptry-associated-protein 1 locus in the sheep parasite *Babesia* sp. BQ1 (Lintan): many conserved features with *B. bigemina*

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Babesia sp. BQ1 (Lintan) is one of the newly described isolates infecting sheep in China and belonging to the *B. motasi* phylogenetic group. In order to develop recombinant sub-unit vaccines to target *Babesia* infecting small ruminant, we focused on RAP-1 (Rhoptry-Associated-Protein 1) as it is known to be involved in the red blood cell invasion process.
rap-1 has been described as a multigenic family of tandemly repeated genes, with proteins sharing common features: 4 conserved cysteines, a signal peptide and several short conserved motifs. We designed degenerated primers from the conserved motifs to amplify a 250 bp central region of the gene and successively sequenced the different tandemly repeated genes. A locus of about 30 kb has been sequenced, including 12 genes and the corresponding intergenic regions. Three types of *rap-1* genes were found: *rap-1a*, *rap-1b* and *rap-1c*, with 6 copies of *rap-1a* intercalated with 5 identical copies of *rap-1b*, and one copy of *rap-1c* located at the 3' end of the locus. Many features of *Babesia* sp. BQ1 *rap-1* locus are closely related to those *of B. bigemina*. The overall organization of the locus is similar, differing by the presence of a *rap-1a* gene upstream *rap-1c* in *Babesia* sp. BQ1 locus, instead of a *rap-1b* in *B. bigemina*'s. All these genes share substantial homologies with *B. bigemina rap-1* genes with nucleotide identities ranging from 63 to 73%. The organization and the sequence of this locus are conserved in the different sheep isolates described in China, namely *Babesia* sp. BQ1 (Ningxian) and *Babesia* sp. Tianzhu.
Standard Reverse Transriptase PCR studies demonstrate that all gene types are transcribed. Antibodies directed against the different RAP-1 proteins were produced from specific synthetic peptides. Protein expression analysis will be presented and the value of RAP-1 as a vaccine candidate will be discussed.

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NcROP40 and NcROP2 based formulation increases pup survival in a pregnant mouse model of neosporosis

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Currently there are no effective methods for the control of bovine neosporosis and many attempts to develop efficacious vaccines have failed. Invasion of the host cell and intra-cellular proliferation of parasites are key events which involve an arsenal of proteins from secretory organelles, and some of these proteins from rhoptries and dense granules have been linked with virulence in *Toxoplasma gondii*. In this respect, NcROP40 and NcNTPase have been shown to be expressed more abundantly in virulent isolates of *Neospora caninum*. Moreover, rNcROP2 has shown positive results when used as vaccine in murine models and NcGRA7 is an immunodominant protein that has been evaluated in different vaccines with varying results. In the present study the safety and efficacy of these recombinant proteins used in single formulations as well as in pair-wise mixtures of rhoptries and dense granules (rNcROP40+rNcROP2 and rNcGRA7+rNcNTPase, respectively) using Quil-A as adjuvant were evaluated in dams and their pups in a pregnant mouse model of *N. caninum* infection. Neonatal survival increased in groups vaccinated with rNcROP40+rNcROP2 (18.2%) and rNcROP2 (6.7%), although only the first one showed statistically significant differences when compared with the rest of the vaccinated groups (0%) ($P<0.0083$, Log-Rank test). Furthermore, average survival time was 11 days for unvaccinated and rNcGRA7+rNcNTPase-vaccinated groups, 12 days for rNcROP40, rNcROP2, rNcGRA7 and rNcNTPase vaccinated groups, and 15 days for rNcROP40+rNcROP2 vaccinated group. These results could be explained by a synergistic effect of both rhoptry proteins. Parasite detection in brains from dams and pups has been also analyzed and results will be presented and discussed. This work has used for the first time a combination of rhoptry antigens as a vaccine and reveals promising results with regard to efficacy.

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Application of reverse vaccinology to the Apicomplexa

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The increasing volume of "omics" data from economically important Apicomplexa, such as *Neospora caninum* and *Eimeria* spp., is now providing an opportunity for the application of an *in silico* approach to the discovery of protein-based vaccines. We are conducting a feasibility study of a proposed high-throughput *in silico* vaccine discovery pipeline constructed from freely available high-throughput prediction programs (Goodswen et al. 2013). These programs are used to gather computational evidence that allows a researcher to make an informed decision as to which proteins, among potentially thousands encoded by an apicomplexan genome, are promising vaccine candidates worthy of laboratory validation. We present an overview of the pipeline and discuss the challenges and pitfalls. The primary challenge is that the evidence is mainly in different independent formats, contradicting, and inaccurate. This considerable uncertainty in the reliability of the evidence arises because it is well acknowledged that an unknown percentage of the ingredients to the pipeline (e.g. protein sequences, database annotations, and predicted evidence itself) are incorrect. We illustrate, using proteins experimentally shown to be potential vaccine candidates, how supervised machine learning algorithms can detect hidden patterns within contradicting and inaccurate evidence that can effectively distinguish true from false vaccine candidates with a sensitivity and specificity of 74% and 83% respectively.

Goodswen SJ, Kennedy PJ, Ellis JT. (2013) A guide to *in silico* vaccine discovery for eukaryotic pathogens. Briefings in Bioinformatics, in press. PMID:23097412

Harmonisation of *in vitro* and *in vivo* models

Dynamics of *Besnoitia besnoiti* infection in the bovine model: answers and questions

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Bovine besnoitiosis is caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti*. Infected cattle are mostly asymptomatic, thus, despite the re-emergence of the disease in Europe, its economic importance is underestimated. Bovine besnoitiosis progresses in two sequential phases: febrile acute or anasarca phase, and chronic or scleroderma phase. Serious consequences of the infection are poor body condition, severe reproductive disorders, particularly in bulls, and eventually death. *B. besnoiti* infection has been described in cattle from observations on naturally-infected field cases, and only a few experimental infections were carried out between the 1960´s and 1980´s. With regard to the parasite developmental stages, both tachyzoites and bradyzoites were shown to be infective for cattle. The definitive host, shedding oocysts and infecting intermediate host (s), remains unknown. It is clear however, that the role of host/parasite dependant factors, which play a major role in the pathogenesis of the disease, needs to be studied in depth. Many fundamental variables are present, such as isolate/strain virulence, dose and the route of parasite inoculation, which were studied under different experimental conditions that makes difficult to compare results. Data on host-dependant factors obtained from naturally infected cattle showed that: i) sero-prevalence of infection is similar in both sexes; ii) there is an increase of seropositive and clinically affected animals with age, although clinical cases in calves were described as well; iii) both beef and dairy cattle are susceptible to the infection; iv) cell mediated immune responses are likely to play an important role since a T-cell response has been detected around several tissue cysts. However, a well-established experimental bovine model that could help to elucidate important questions is not available to date. The critical questions on whether venereal and/or transplacental transmission takes place; whether chronically unapparent infected cattle are resistant to re-infection; whether colostral antibodies are protective; to what extent humoral response of the host might reflect the disease/protection status require further research. An update on clinical and pathological findings including our own unpublished data will be presented and discussed.

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Vaccination of pigs and lambs against *Toxoplasma gondii* reduces tissue cyst formation; safer meat for human consumption

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The protozoan parasite *Toxoplasma gondii* is a zoonotic pathogen that has the ability to infect all warm blooded animals including humans. Toxoplasmosis is a major opportunistic disease of immunocompromised patients. It also represents a serious threat during pregnancy, causing severe foetal abnormalities or potentially leads to problems in childhood or later adult life. Undercooked or raw meat containing infective tissue cysts are a significant source of human infection. The production of *T. gondii* tissue cyst free meat could reduce the risk of human exposure to *T. gondii*.

In two different animal studies a group of 23 pigs and 32 lambs were used to determine the efficacy of a commercially available vaccine that protects sheep from abortions caused by *T. gondii*, with an aim to reduce tissue cyst formation. Following vaccination, animals were challenged with oocysts. Subsequently a mouse bioassay, using a variety of porcine tissues, resulted in a 100% survival of mice that received tissues from vaccinated / challenged pigs, while bioassays of tissues from non-vaccinated pigs resulted in a survival rate of 51%. Parasite DNA was also identified in the homogenate used in bioassays from the non-vaccinated / challenged group but not in the vaccinated/challenged pigs. In a similar vaccination study in sheep, *T. gondii* DNA was tested for in their tissues. Following vaccination and challenge with 100,000 oocysts of the Moredun M4 strain, the parasite was detected at significantly lower levels in heart and skeletal muscle samples from the vaccinated/challenged group (0% and 5.9% respectively), when compared to the non-vaccinated/challenged animals (75% heart, 87.9% skeletal muscle).

The results demonstrate that vaccination of pigs and lambs with the S48 attenuated *T. gondii* strain can reduce the formation of tissue cysts, resulting in potentially safer meat for human consumption.

Occurrence of acute phase abortions in an experimental infection with *Toxoplasma gondii* in sheep

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In order to study the pathogenesis of abortion due to ovine toxoplasmosis, an experimental infection of pregnant sheep at midgestation and weekly serial culling was designed. After inoculation of ewes with 2000 freshly prepared sporulated oocysts of *T. gondii* strain M4, unexpected abortions occurred between days 7 and 11 post infection in all pregnant and infected ewes. Unexpected abortions happened again between days 9 and 11 post infection in 58% of the infected ewes when they where orally infected at late gestation with 500 oocysts, while classical abortions in natural and experimental ovine toxoplasmosis usually occur one month after infection. Few experimental studies have reported the so-called acute phase abortions as early as twelve days after oral inoculation of oocysts. In those studies, the underline mechanism for abortion was not elucidated, although pyrexia was proposed to be the trigger for abortion. In the present study, body temperature curves or kinetics of serological antibodies shown by the aborting sheep were not different from those found in infected sheep not suffering acute phase abortion or reported in previous experiments. All studied placentas from ewes suffering acute phase abortions showed infarcts and thrombosis in the caruncullar villi of the placentomes and ischemic lesions (periventricular leukomalacia) in the brain of some fetuses. The parasite was not identified by PCR in samples from the foetus and only in placentomes. Furthermore, no antigen was detected by immunohistochemical labeling of any foetal or placental sample. Although not statistically significant, ewes suffering acute phase abortion showed an early production of peripheral IFNγ when compared with infected non-aborting ewes. These findings suggest that the vascular lesions found in the placenta, and the consequent hypoxic damaged to the foetus, could be associated to the occurrence of acute phase abortions and also that the relevance of *T. gondii* as cause of sheep abortion may be underestimated under routinely sampling and analysis.

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An advanced in ovo model for *Cryptosporidium baileyi*

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Infections of chicken eggs with *Cryptosporidium baileyi* have been conducted previously. Regarding current needs for screening systems, *in ovo* models offer easy handling and are inexpensive in comparison to more complex animal models. Factors such as animal husbandry facilities, animal attendants and costs for maintenance can be reduced to a minimum or completely neglected in this model.

The crucial step for establishing reproducible *in ovo* infection systems for *Cryptosporidium* sp. was the evaluation of the optimal infection time and its influence on the parasite output, as well as necessary parasite pretreatment and sample collection.

Furthermore, the gained results were evaluated for a drug model application. The presented *in ovo* system is a valuable and highly sensitive model for *Cryptosporidium* infection studies *in vivo* with the potential to be used as a drug model. Another advantage of the method is the possibility of simultaneous monitoring of toxic effects due to drug administration in the chicken embryo. Thus, drug screening and first parts of a toxicological screening are efficiently combined.

Implementation of the *Babesia divergens* transmission model: an essential tool to study *Babesia*-tick molecular interactions

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Babesiosis, a zoonosis caused by the apicomplexan protozoa of the genus *Babesia*, is a tick-borne malaria-like disease of various vertebrate hosts. Despite a growing attention paid to *Babesia* and babesiosis as an emerging disease in both veterinary and human medicine, the knowledge of interactions of *Babesia* and tick as a vector is still insufficient. To this end, no research in this area has ever been focused on the transmission model of the most common European species - *Babesia divergens* and its tick vector *Ixodes ricinus*. This is particularly due to the absence of a suitable laboratory host that could be used for the infection of tick adult females and hereby simulate a natural transmission cycle of *B. divergens*. In order to overcome this obstacle, we implemented an artificial feeding technique of *I. ricinus* females on *in vitro* culture of *B. divergens* in bovine red blood cells. The infectious *in vitro* feeding opens a gate towards detailed investigation of *B. divergens* development cycle as well as to identification and characterization of molecules playing a role in transmission and persistence of the parasite within the vector. Detailed knowledge of the *Babesia* life stages in the tick vector together with the knowledge of *Babesia* transmission mechanisms from the vector to the vertebrate host can significantly contribute to the control of babesiosis in Europe, whether human or bovine.

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Neospora caninum experimental infection in pregnant sheep as a model for exogenous transplacental transmission for ruminant neosporosis

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The availability of well-defined ruminant models of infection is one key factor for studying the pathogenesis and to evaluate vaccine candidates and therapeutics in bovine neosporosis. Although laboratory (mice) models have been broadly employed, due to the evident differences between mice and ruminants, results obtained could not be always extrapolated to cattle. Sheep has several advantages over cattle as an experimental animal model including size, length of gestation and cost. Furthermore, recently the awareness and importance of *Neospora caninum* as an abortifacient in small ruminants has increased. The aim of this work was to investigate experimental infection by *N. caninum* in pregnant sheep as a valid model for exogenous transplacental transmission for ruminant neosporosis. Thus, pregnant ewes were intravenously inoculated at days 40, 90 and 120 of gestation, with 10^6 tachyzoites of Nc-Spain7 isolate. Infection during first and second third of gestation resulted in abortion of all foetuses, although it happened earlier at day 40 (19-21 days post-infection) than at day 90 (34-48 days post-infection). Lambs born from ewes infected during the third gestation period were all viable, although some of them were very weak. Placentas and foetuses from all infected ewes showed histological lesions characteristic of neosporosis. In the foetuses, lesions were found mainly in liver and lung while they become more frequent in brain and skeletal muscle as the pregnancy advanced. In general, lesions were mainly necrotic in foetal samples from ewes infected in the first trimester and showed more conspicuous infiltration of inflammatory cells on those infected in the second and last trimester. The results obtained so far show that the infection had a similar outcome in experimentally inoculated sheep to that described in natural bovine or ovine neosporosis.

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Development of an *in vitro* germ carrier assay to test disinfectants against parasitic permanent stages

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Cryptosporidium parvum oozysts are highly resistant against many disinfectants and environmental conditions. Therefore they are very useful as surrogate for the testing of disinfections against dispersal stages of other coccidia. We developed an assay that combines germ carrier, cell culture and quantitative PCR to assess the effectiveness of disinfectants under standardised conditions. As germ carriers we used brushed stainless steel disks. Oocysts were dried on these discs and subsequently treated with different disinfectants. Oocysts were then used to infect human ileocecal colorectal adenocarcinoma (HCT-8) cells. The number of Parasites that had infected the cells was assessed by quantitative PCR.

Treatment of oocysts with cresol based disinfectants showed a dose dependent reduction in infectivity. A concentration of 2% cresolic disinfectants was already enough to inactivate more than 98% of the oocysts. As internal standard we used heat inactivated oocysts (70°C for 20 min) what leads to a 99.9% inactivation.

The presented method gave reproducible results and seems therefore suitable to replace the *in vivo* chicken test with *Eimeria tenella* that is the only test system recommended by the DVG (German Veterinary Medical Society) at the moment.

Genomics and proteomics

Genomic analysis of the causative agents of coccidiosis in chickens

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Seven species of *Eimeria* are known to infect poultry and cost industry around £2 billion annually (Shirley et al. 2005). Drug resistance evolves rapidly in these species and thus there is a drive to develop novel therapies (Blake et al. 2011). To provide a basis for better understanding of *Eimeria* biology we have sequenced the genomes of all seven species infecting chicken and characterized the transcriptome of several key life stages of the most important veterinary species *Eimeria tenella* at base-pair resolution. Using these resources we address various aspects of *Eimeria* biology and expand our understanding of the Coccidia (the largest group of Apicomplexans) and their relationship to other Apicomplexa such as *Plasmodium*. In particular we (1) characterize the surface antigen gene family (sag) in *Eimeria*, across all seven species infecting chicken, (2) show that *Eimeria* genomes are the most extreme seen thus far in terms of protein-coding repeats and (3) characterize a family of retrotransposons in *Eimeria* species which may have been acquired by horizontal transfer from the ancestor of the apicoplast, a plastid present in all Apicomplexa.

Filling the gaps: expression profiling in *Toxoplasma* sexual stages

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The intestinal development of the zoonotic apicomplexan parasite *Toxoplasma gondii* in cats has remained understudied because of the highly limited access to these stages within infected animals and the absence of an *in vitro* culture system. While the coccidial life cycle of *Toxoplasma* is confined to intestinal epithelial cells of felids, asexually proliferating stages also establish systemic infections in cats and in a wide range of intermediate hosts including humans. These extraintestinal stages of *Toxoplasma* are easily cultivated *in vitro* and amenable to a vast range of experimentation including forward and reverse genetics. During the past two decades this provided an exceptional opportunity to discover many aspects of the Apicomplexa-specific molecular machinery responsible for host-parasite interaction. Because of the recent massive increase in sequencing and computing power the hitherto sequestered enteroepithelial stages are now also becoming accessible to molecular analysis. Filling these vast knowledge gaps and drafting a new chapter in research on coccidia and other Apicomplexa has come within reach thanks to complete pipelines for gene expression profiling of parasites isolated from experimentally infected animals. Although almost all knowledge about Apicomplexa biology is currently about cultivatable extraintestinal stages, this information now proves invaluable by providing a multitude of biological reference points for the interpretation of new gene expression data and a scaffold for the rapid construction of a molecular road map of the coccidial cycle of *Toxoplasma*.

Reporter gene strategies illuminating evolutionary conserved oocyst wall formation in *Eimeria*

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An important key 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 specificity of this process. To illuminate the fate of proteins involved in oocyst wall formation, reporter gene assays are a valuable tool.

A reporter gene system with plasmids containing a multifunctional *dhfr-tsm2m3-yfp* yellow fluorescent pyrimethamine resistance gene and a reporter gene encoding for the oocyst wall protein *etgam56* from *Eimeria tenella* has been developed.

To provide stage specific reporter signals *etgam56* was fused with *mcherry* and controlled by the *Eimeria tenella* gamogony gene specific regulatory sequence of *etgam56*. The red fluorescent mCherry was inserted into different parts of the *etgam56* sequence to confer site specific information of potentially processed peptides. With this approach it was possible to follow processes of oocyst wall formation in living *Eimeria nieschulzi* parasites for the first time.

Genome analysis of two genotypes of *Theileria orientalis* isolated in Japan and comparative genomics with lymphocyte-transforming *Theileria* species

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Theileria orientalis, a tick-borne apicomplexan parasite of cattle is widely distributed in the world, and generally believed to cause benign diseases compared to diseases caused by lymphocyte-transforming *Theileria* species such as *T. parva* and *T. annulata*. However, *T. orientalis* can cause anemia, icterus, and even death in severe cases, which produces economic loss in grazing cattle of Japan and South Korea. In addition, serious diseases caused by *T. orientalis* have been recently recognized in Australia and New Zealand.

There are several genotypes of this parasite species distinguishable by major piroplasm surface protein gene sequences and 16s rDNA. Among them, two genotypes, Chitose (Type I) and Ikeda (Type II) are dominant in cattle with clinical symptoms, and the infection with the latter type is usually associated with more severe clinical cases. Recently, we reported the genome sequence of *T. orientalis* Ikeda type (Hayashida et al. 2012), and we compare it with the genome of Chitose type in this presentation. In addition, their genomic features were compared with those of *T. parva*/*T. annulata*. The nuclear genomes of both types of *T. orientalis* were approximately 9.0 Mbp in size, which are larger by 8% than those of *T. parva* and *T. annulata*. Overall syntenies of three *Theileria* species are well conserved while subtelomeric structures differed dramatically, even within the two *T. orientalis* genotypes. Gene families specific for transforming *Theileria* parasites were identified in order to search genes responsible for host cell immortalization. The orthologous genes encoding surface antigens found in transforming *Theileria* species, such as p67/SPAG (sporozoite stage) and PIM (schizont stage), were identified in *T. orientalis*, which may facilitate vaccine development for “oriental” theileriosis. Our comparative genomic studies between *Theileria* species and within "benign" species with different pathogenicity will provide insight into the unique biological properties of *Theileria* parasites.

Genome wide expression profiling for identification of stage regulated transcription factors and binding motifs in *Theileria annulata*

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Stage differentiation from one life cycle stage to the next is an event that is critical for propagation and transmission of apicomplexan parastites. Certain differentiation steps are conserved across many genera: the generation of invasive merozoites from a multinucleated schizont, for example. These similarities indicate that the differentiation mechanism(s) will show a degree of conservation. Previous work on *Theileria annulata* established an *in vitro* system of differentiation from the multinucleated macroschizont to the uninucleated merozoite stage (merogony). Analysis of this system established that differentiation was stochastic (asynchronous with the probability of a differentiation event occurring influenced by altered culture conditions and cell lineage). A model was proposed whereby stage differentiation operates on the basis of regulators of gene expression reaching a commitment threshold. It was further postulated that commitment is reached via an initial reversible increase in factor level relative to its nucleic acid template (growth versus division). In this study we have utilised microarray analysis to expression profile the genome of *T. annulata* from the sporozoite stage through merogony to the piroplasm stage that is transmitted to ticks. Groups of genes showing significant alteration in the profile of expression were then used to screen for putative transcription factors/DNA binding proteins and nucleotide motifs present in intergenic regions. The results indicate the presence of both DNA binding proteins (ApiAp2) and motifs that could operate in a stochastic model of differentiation and show conservation across the apicomplexa.

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A tale of two algal genomes: evolution of parasitism in apicomplexans

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The genomics era of apicomplexan parasites had started with the publication of two *Plasmodium* genomes over a decade ago. Since then, at least 15 different species of Apicomplexan parasite genomes have been sequenced, with many more in the process. These studies have revealed an unprecendented amount of information on the genome and the biology of this group of versatile human and animal parasites with diverse life styles and host tropism. However, a key question remains unsolved: how could apicomplexan species be so successful in becoming parasites among diverse hosts? To explore this, we have analysed draft nuclear genome sequences of two chromerid species: *Chromera velia* and *Vitrella brassicaformis*, a group of free-living alga with ancestral relationship to the apicomplexan parasites. Comparative genome analyses show that the core apicomplexan genes, which are conserved among the majority of apicomplexan parasites, are highly conserved in chromerids. We found evidence for both ancient and lineage-specific evolution of gene families. Together, these suggest that the primitive molecular toolkits of parasites were initially gained before split of chromerid and apicomplexan species, which was further evolved to adapt to specific host environments. We will be presenting highlights of our analyses.

Recent insights into the molecular phylogeny of piroplasmids

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Piroplasmids are tick-transmitted hemoprotozooans which infect mammals and birds. They are acknowledged for their major impact on farm and pet animal health and associated economic costs worldwide. Piroplasmids represent an apicomplexan group that consists of the closely related *Babesia*, *Cytauxzoon* and *Theileria* parasites. Based on a phylogenetic tree of all currently available 18S rRNA genes, we present a thoroughly revised molecular classification, comprising five monophyletic *Babesia* lineages, one *Cytauxzoon* clade, and one *Theileria* clade. Notably, *Babesia* lineages constitute a paraphyletic assemblage calling for a change of genus name for at least four of these lineages. Monophyly of *Cytauxzoon* species could be confirmed as all segregated in a single clade. According to their tree placement *Theileria youngi* and *T. bicornis* represent *Babesia* species. Importantly, the previously unresolved tree placement of *T. equi* (formerly: *Babesia equi*) could be established and suggests that *T. equi* is neither a *Theileria* nor a *Babesia* species but belongs to a separate piroplasmid clade. Human babesiosis, also transmitted by blood transfusion, is an increasing public health concern. Remarkably, at least seven different *Babesia* species belonging to three different unrelated clades have been reported to infect humans. So far, three *Babesia* species infecting birds have been reported and each represents a sister taxon to other *Babesia* clades: *B. kiwiensis* (representing a sister taxon to a clade including *Babesia* parasites infecting carnivors), *B. bennetti* (repersents a sister taxon to *Babesia* s.s.) and *B. poelea* (representing a sister taxon to the *Babesia* "western clade"). This suggests that *Babesia* species infecting birds played an important role in the dissemination of the parasite at an early timeframe of piroplasmid evolution. A more widespread taxon sampling across all potential vertebrate and tick hosts as well as the construction of trees using multiple genes will allow to further advance *Babesia* phylogeny and taxonomy.

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The 'shaveome' of *Babesia bovis* infected-red blood cells

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Babesia bovis is an important pathogen of economic significance to the beef and dairy industry worldwide. The precise molecular mechanisms underlying the pathophysiology in susceptible cattle remain unknown, however, it is clear that a large component of severe disease occurs as a result of parasite-induced modifications to the normal structure and function of the red blood cells (RBCs) in which the parasites reside. Importantly, these cellular changes are underpinned by the export of numerous, currently uncharacterised proteins from the parasite and into the host RBC. We have used proteomic and bioinformatic approaches to identify important parasite proteins that are likely to contribute to the pathogenesis of bovine babesiosis. Our recent studies have focussed on the identification of parasite proteins that are exposed on the surface of the infected RBC which are likely to be involved in mediating the interaction between parasite-infected RBCc and the host vasculature and in evasion of the hosts' immune response. Using proteases to 'shave' the outer surface of infected RBCs followed by analysis using LC/MS/MS, we have identified a subset of 40 *Babesia*-encoded proteins, which are likely to represent the surface proteome of *B. bovis*-infected RBCs. We have also experimentally verified by immunofluorescence analysis that some of these proteins do in fact exist on the surface of infected RBCs and suggest that these may represent new therapeutic targets for the development of next-generation vaccines for babesiosis.

The genome of *Eimeria falciformis* allows identification of conserved invasion strategies but diverged host interaction in the Coccidia (Apicomplexa)

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We present the complete genome sequence of the intracellular mouse parasite *Eimeria falciformis.* The genus *Eimeria* is the largest and most diverse genus of apicomplexan parasites. It comprises more than 1,800 species, some of which are important pathogens of domestic animals. The genome of *E. falciformis* is 44 Mb in size and contains 5,879 predicted protein coding genes. Comparative analysis of *E. falciformis* with *Toxoplasma gondii* shows an emergence and diversification of gene families associated with motility and invasion mainly at the class level, the Coccida. Many rhoptry kinases, among them important virulence factors in *T. gondii*, are absent from the *E. falciformis* genome. Surface antigens are divergent between *Eimeria* species and differences in mannose metabolism, GPI-anchor and N-glycan synthesis are found relative to *T. gondii*. *E. falciformis* possesses a reduced set of transmembrane transporters and we predict an altered mode of iron uptake in the genus *Eimeria*. Reduced diversity of genes required for host-parasite interaction and transmembrane transport and potentially a restricted metabolic potential allow hypotheses on host adaptation and specialization. The *E. falciformis* genome sequence sheds light on the evolution of the Coccidia and helps to establish *E. falciformis* as a useful model-parasite.

Posters

Apicomplexa in camels of Central Iran

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Dromedary camels (*Camelus dromedarius*) are important multipurpose animals of arid and semiarid parts of the world. In Iran they are mainly kept for the purpose of meat production. According to the last enumeration, 153000 camels live in Iran, and 21690 of them (about 14%) have been counted in Yazd province in center of the country. Considering the need for epizootological studies in the region, twelve research projects were conducted in joint cooperations of Ahvaz, Yazd and Urmia Universities from June 2008 to September 2010. Most of those studies were done on camels' infectious diseases of zoonotic importance.
Totally 395 blood, 350 fecal, 150 abomasal mucusa and 780 meat samples were taken for parasitological, bacteriological and virological studies. The recovered apicomplexan parasites, applied methods and infection rates are summarized in the table below.

Agent	Infection rate	Method
Piroplasms	15.79%	Light microscopic assay (Giemsa stain)
Cryptosporidium	20.33% of feces	Light microscopic assay (Modified Ziehl-Neelsen stain)
	12% of abomasal mucusa	
Eimeria	9.51%	Saturated zinc sulfate flotation technique; Light microscopic assay
Toxoplasma gondii	14.56%	Serologic (modified agglutination test, MAT) pretreated with 2-mercapto ethanol
	No positive blood sample	Polymerase chain reaction (PCR)
Neospora caninum	3.94%	Serologic (*Neospora* agglutination test, NAT) pretreated with 2-mercapto ethanol
Sarcocystis	No macroscopic cyst	Naked eye inspection
	51.5%	Pepsin digestion method; Light microscopic obs. of Giemsa stained slides

Results of those studies show considerable rates of zoonotic agents' infections in camels of the study area. Therefore, control and eradication strategies for prevention of spreading in animal and human populations seem necessary. Also, further molecular studies for determination of exact taxonomic status of apicomplexan parasites of camelids must be conducted.

Molecular detection of apicomplexan parasites in Hatay (Antioch), Turkey

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The majority of the tick-borne apicomplexan blood parasites have different host species like wild mammals, birds and reptiles which are all important in epidemiology. Some of the common vector ticks have different reservoirs and hosts in the wildlife. The role of vector ticks and wildlife reservoirs on sustainable herd breeding, is important to the inference of patterns of pathogen's genetic shift/drift flow.

In the first part of this study, wild ruminants *Gazella gazella, Capreolus capreolus*, *Capra aegagrus* and domestic herd animals (cattle, goat, sheep) and shepherd dogs were sampled for molecular detection and phylogenetic analysis of tick borne apicomplexan blood parasites (*Babesia* sp, *Theileria* sp, *Hepatozoon* sp) The Ricketsial blood pathogens (*Anaplasma* sp, *Ehrlichia* sp.) and *Mycoplasma* species (*Haemobartonella* sp. in old) were also investigated. New reports about the tcik borne apicomplexan species were recorded from the wild life in Hatay. Determination of apicomplexan genetic differences between wild and domestic life, ticks and reservoirs, hosts and intermediate hosts are ongoing.

There is a common survival among the Hatay region just on the border line. The Orontes River is a natural biobridge between Turkey and Syria. Movement of vectors, reservoirs and infected hosts among this biocorridor may influence the dispersal of drug resistance alleles. The impact of point is related with virulence of pathogens and efficiency of vaccines. Also understanding the genetic variation of parasites is important for implementing successful wildlife control programs and prevention of diseases in domestic herds.

Molecular identification and diagnosis of an apicomplexan parasite forming macroscopic cysts in the muscles of South American camelids

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The domestic South American camelids (SAC) llama and alpaca constitute the main livestock species in the Andean regions. Their meat is an important source of animal protein for rural families and a product of growing interest for local and international gourmet cuisine. Abundant macroscopic cysts (1-5 mm) are a frequent finding in skeletal muscles and render the meat unsuitable for commercialization, with the consequent economic loss for SAC small-scale breeders. We observed that cysts contain an average of 2.2 x 10^7 half-moon shaped cells of oscillating motility and a medium length and width of 17.6 and 3.6 µm, respectively. These observations are consistent with *Sarcocystis* sp. bradyzoites. To verify the parasite species, the whole 18S rRNA gene was PCR-amplified and sequenced from genomic DNA isolated from cyst samples of three llamas from the Andean flatlands of Argentina. A phylogenetic tree based on a Bayesian analysis was constructed including available *Sarcocystis* spp. 18S rRNA sequences. The three 18S rRNA gene sequences of llama isolates segregated in a single clade, together with the sequence of *S. aucheniae*, reported from an alpaca in Australia. After confirmation of the parasite species as *S. aucheniae*, a semi-nested PCR based on the 18S rRNA hypervariable region was set up. The test was able to amplify the genomic DNA of 0.1 parasite (corresponding to a parasitemia of 100 protozoa/ml blood), as tested in llama blood spiked with known amounts of *S. aucheniae* bradyzoites. Importantly, this PCR detected natural *S. aucheniae*-infections in blood samples of llamas, as corroborated by amplicon sequencing. Molecular detection of *S. aucheniae* in live SAC can significantly contribute to an improved management of herds and to future sarcocystiosis eradication campaigns.

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The relationship between seropositivity and tissue cysts in sheep naturally infected with *Toxoplasma gondii*

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Toxoplasma gondii has three consecutive infectious stages including; tachyzoite, bradyzoite and sporozoite. After the infection, during the chronic phase of toxoplasmosis, tissue cysts can develop in different tissues of the hosts. Tissue cysts of *T. gondii* are about 5-70 µm in diameter, harbour a number of bradyzoites that are resistant to gastric digestion and responsible for foodborne transmission to the intermediate hosts. People are infected by consumption of raw or undercooked various animal tissues harbouring the tissue cysts. Most farm animals are seropositive for *T. gondii.* Seropositivity shows whether a host is infected with *T. gondii*, however, it fails to determine the presence of infectious tissue cysts. Thus, understanding the relation of seropositivity with presence of the tissue cysts in meats originating from seropositive sheep offered for human consumption has extreme importance for the epidemiology of toxoplasmosis in humans. With the present study, we aimed to determine the relationship between the seropositivity and the presence of tissue cysts in skeletal muscles and brains of sheep.

In this study, skeletal muscles (tongue, masseter, leg, intercostal and diaphragmatic muscles) and brain samples of 100 sheep at slaughter were analysed for the presence of *T. gondii* tissue cysts along with serum IgG titers. Two methods of isolation by percoll gradient centrifugation and tissue microarray (TMA) technique with immunoperoxidase staining were used. Seropositivity was detected in 88% (88/100) of sheep serums analysed by IFAT and SFDT and tissue cysts were observed in 46 (52.2%) and 15 (17%) of the seropositive sheep with isolation technique and TMA-immunoperoxidase staining, respectively. The diameters of the tissue cysts were measured to be 25-58 x 25-62 (mean 34 x 36) micrometer. The relationship between the presence of the tissue cysts and the seropositivity in sheep was found statistically significant at 1/16 ($p<0.01$) and at 1/64, 1/128 ($p<0.001$) serum dilutions.

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Bovine besnoitiosis in Croatia

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Bovine besnoitiosis is re-emerginig disease in Europe caused by cyst forming apicomplexan parasite *Besnoitia besnoiti*. Today bovine besnoitiasis is endemic in some regions of Spain, France, Porugal and recently Italy, whilw imported cases have been dected in Germany and Switzerland. During the late winter 2012/2013 workers observed inflammation and irritation of skin in bull of Aubrac breed, French beef breed imported to Croatia in 2010, together with two more bulls, 60 heifers, 6 cows and 6 calves from France. A new group of cattle including a bull and 66 heifers were imported in 2011. Soon after the scrotum skin begun to look like oak crust, other symptoms also appeared, like lower limb swelling, hind legs staggering. Following the local veterinarian prescription (diagnosis of mange) the animal was treated with ivermectin, corticosteroids and antibiotics but without success. In skin sample taken from the hind leg near inguinal region we have microscopically determined cysts with bradyzoits that corresponed to *Besnoitia besnoiti*. Besnoitiasis was confirmed with histopathology, PCR and subsequent sequencing. To investigate the prevalence, all cattle from the farm were checked for presence of antibodies to *B. besnoiti*. Antibodies for *B. besnoiti* were identified in 42.3% (66/156) out of 156 individual sera samples. In the first group of imported cattle antibodies were detected in 50% (65/130); 48% cows (61/126) and all bulls (4/4), while in heifers borne on the farm (26) single animal was infected (4%). Cysts were present in vulva or/and sclera in 37% of sero-positive animals. Here, we have confirmed introduction of *B. besnoiti* in Croatia after import of infected cattle from endemic region of France and first detection outside Western Europe. This finding confirmed that main way of spreading of disease is import from endemic region and that further spreding can happen without known final host.

Prevalence and geographic distribution of bovines seropositive for *Besnoitia besnoiti* in Portugal

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Bovine besnoitiosis was recently considered an emergent disease in Europe, due to the increasing prevalence and geographical expansion in several countries. In Portugal, various outbreaks have been recorded since 1991 in the region Alentejo, but data on prevalence and geographic distribution of infection are absent. The objective of this study was to estimate the prevalence and geographic distribution of animals infected with *B. besnoiti* in Portugal through a nationwide seroprevalence study.
A total of 13020 animals were randomly selected and tested for the presence of specific anti- *B. besnoiti* antibodies. Sera were obtained from 402 herds distributed over 88 municipalities and covered the five NUTS II regions of Portugal (North, Center, Lisbon, Alentejo and Algarve). The serological status of animals was determined by a serial testing strategy, using the modified agglutination test (B-MAT) as the first screening assay and the IFAT as the confirmatory test.
After adjustment for herd size overall prevalence was calculated to be 2.8% (95%CI: 2.1%-3.6%). Positive animals were identified in 17 municipalities (19.3%), of which 12 are located in the Alentejo (44.4% of the herds tested in this region), 2 in the Center (7.4%), 1 in Lisbon (33.3%) and 2 in the North (6.7%). Within-herd prevalence varied between 0.7-72.4%. Spatial scan statistics analysis identified two spatial clusters: one primary cluster covering the upper Alentejo and a secondary cluster in the northeast of Portugal. Seroprevalence at animal and herd level was significantly higher in Alentejo, compared to the other NUTS II regions ($p<0.0001$). Antibody prevalence was also significantly higher in the age group >48 months ($p<0.0001$), while gender differences were not significant ($P>0.05$).
Further epidemiological research is needed to identify eco-geographic factors, which could explain the primary clustering of *B. besnoiti* in the Alentejo region.

NOT PRESENTED

Considerations on the control of bovine besnoitiosis in Europe

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Besnoitiosis has been described in cattle late XIX century in France and Portugal, being from the beginning of the XX century an absent or silent disease. In the beginning of the eighties from the XX century the disease had been reported in Portugal and Spain, being almost on the same time, reported and spreading throughout France. Recently was reported in Germany, Switzerland and Italy, being considered an emergent disease in Europe by EFSA in 2010. This disease, without any known treatment and, which biological cycle is no known (no definitive host has been identified), causes in both acute and chronic bovine besnoitiosis significant economic constraint to commercial cattle production. In the acute stage of disease, parasitic tachyzoites invade blood vessels of the skin, subcutaneous tissues, fascia and testes causing widespread vasculitis and thrombosis. The result is a severe generalized systemic reaction which is accompanied by edema of the skin and acute orchitis. During the chronic stages of disease, large numbers of tissue microcysts containing bradyzoites are formed. The most striking features of this stage are thickening, wrinkling and hair loss of the skin, accompanied by anorexia and severe weight loss. The case fatality rate is approximately 10%. Irreversible male sterility or impaired fertility is a common sequela in breeding bulls, and is one of the most negative aspects of the disease in animals that survive the acute and chronic stages of infection.
In the present work, besides a review of the disease in Europe, there is made an overview on the control measures being used in Europe for the control of the disease.

Investigation of *Theileria equi* and *Babesia caballi* by Real Time PCR in horses in Karacabey Region of Bursa and molecular characterization of the isolates

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This study was carried out to determine the molecular prevalence and characterization of *Theileria equi* and *Babesia caballi* in horses in Karacabey region of Bursa and supported by Erciyes University Scientific Research Fund with the project number TSD-10-2876. Between May and July of 2009, blood samples were collected from totally 203 horses, in which 153 were belong to Agricultural Management Directorate and 50 were belong to Turkish Jockey Club Karacabey Stud Farm. Following genomic DNA extractions from blood samples, TaqMan probe based Real Time PCR were performed with the specific primers which partially amplified the 18S rRNA gene region of *B. caballi* and *T. equi*. 10 (4.93%) of the examined horses were found to be infected with piroplasmosis. When examining the distribution of the positive samples over the collection centers, 4 and 3 horses from Agricultural Management Directorate and 2 and 1 horses from Turkish Jockey Club were found to be positive for *T. equi* and *B. caballi,* respectively. The prevalence of *T. equi* and *B. caballi* in the research area were determined as 2.96% and 1.97%, respectively. According to the conventional PCR which partially amplifies the 18S rRNA gene region, totally 5 isolates, 4 from *T. equi* and 1 from *B. caballi*, were determined to give amplification on the agarose gel. Conventional PCR showed 50.0% sensitivity and 100.0% specificity when compared with the Real Time PCR. Among the PCR positive isolates only two *T. equi* isolates (BK-1, BK-2) were found to have suitable concentration for sequence analyses. Based on 18S rRNA gene sequences, BK-1 and BK-2 *T. equi* isolates obtained from horses of Bursa Karacabey region were determined to be 100.0% identical to each other and showed 2.5±0.6% genetic distance from other *T. equi* isolates available in GenBank and also they were determined in the *T. equi* Genotype E.

Identification of a novel and zoonotic GP60 subtypes of *Cryptosporidium parvum* from calves in dairy areas of Argentina

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Cryptosporidium spp. infections are an important cause of gastrointestinal disease worldwide in a variety of hosts, including humans and cattle. In Argentina and many other countries cryptosporidiosis is responsible for significant fatalities of neonatal calves, resulting in substantial economic loss in dairy farming. *C. parvum* represents the main zoonotic species and calves are considered a major host reservoir. Molecular epidemiological tools have allowed much progress in the understanding of transmission routes. However, current reports are considerably biased towards relatively few industrialized countries and knowledge on the circulating species and subtypes involved in bovine cryptosporidiosis in Argentina are still lacking. In order to achieve a better understanding of the molecular epidemiology of *Cryptosporium* spp. in calves species identification based on the small-subunit rRNA and subtyping using the GP60 gene were carried out. Fecal samples were collected from calves aged less than two months old located in the main productive dairy region of Argentina, *Cryptosporidium* sp. oocysts were microscopically detected in stained smears, and positive samples were further analyzed. Exclusively, the *C. parvum* species was identified in the study group. Subtyping and phylogenetic analysis of the *C. parvum* GP60 gen revealed the presence of altogether 6 alleles, all belonging to the IIa family. Among them, one (IIaA23G1R1) represents a novel subtype and four (IIa18G1R1, IIaA20G1R1, IIaA21G1R1, and IIaA22G1R1) have been recognized in relatively few studies and/or with low frequencies worldwide. Importantly, subtype IIaA17G1R1, which is strongly implicated in zoonotic transmission, was also identified in this study, suggesting that calves may represent a potential source for human cryptosporidiosis in Argentina.

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Novel Nested-PCR method for *Babesia ovis* identification on blood and tick samples from small ruminants

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Babesia ovis is a tick-transmitted protozoa parasite that infects small ruminants and causes host-mediated pathology and erythrocyte lysis. Common in tropical and sub-tropical areas, the presence of this parasite has an economical impact on industry and for that reason sensitive methods of diagnostic are required. Resorting for molecular biology techniques, conventional Polymerase chain reaction (PCR) is the most used for *Babesia* detection on biological samples, however some false positive cases are common due lack in sensitivity. Therefore a Nested PCR was developed and consequent reaction conditions were optimized and tested with field samples. Both pairs of primers were designed considering the small subunit of 18S rRNA sequence already published on Genbank.

Blood and tick samples from small ruminants were collected from different regions of Portugal and DNA extraction were performed using commercial kits. In the particular case of ticks, each one was analyzed in terms of gender, specie and mouth integrity and homogenized before DNA extraction.

After DNA quantification and dilution, conventional PCR and Nested-PCR were accomplished for all samples. Appealing for an agarose gel electrophoresis, the results from both methodologies were compared and the sensibility of Nested PCR was evaluated. Employing the Nested-PCR, it was possible to identify a higher number of positive cases among the evaluated samples. The validation of the results was achieved by sequencing the obtained DNA fragments. The diagnostic method here described is at this moment the most effective and sensitive for *Babesia ovis*' detection in field blood samples and ticks.

Survey of *Theileria* infection prevalence in sheep of Piranshahr city, Iran

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Sheep and goat breeding is one the most significant source of domestic husbandry industry in Iran. Each year a significant number of animals produced in the country are removed for various reasons, and among these are the parasitic diseases of small ruminants.

Theileriosis is an important hemoparasitic disease in small ruminants in the tropical and subtropical regions of the world and causes every year a large economic losse to livestock breeder's imports. Two species of *Theileria*, *T. lestoquardi* and *T.ovis* cause ovine theileriosis in Iran. Malignant ovine theileriosis (MOT) is transmitted by the vector tick *Hyalomma anatolicum anatolicum*.

In the current study sheep from Piranshahr city, in the West Azarbiejan province, North-west of Iran, are investigate. 260 samples were selected randomly during the 4 seasons. Blood smears were collected at marginal ear veins and samples were fixed in methanol, stained by Giemsa solution and examined by microscopy. Results were analyzed by SPSS software.

From a total of 260 sample, 4 (1.54%) positive were observed from which 3 (75%) were females sheep and 1 (25%) were male; 2 (50%) were animals under one year and 2 (50%) were animals between 1-3 years old. We did not observe any significant differences between the rate of *Theileria* spp. infection of all the age and sex groups of the sheep examined ($P > 0.05$).

Theileria parasites in Oman

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Theileriosis is a widespread protozoan tick-borne disease of ruminants in Oman, causing high morbidity and mortality. However, little is known about the parasite species, their pathogenicity and prevalence. The present study examined Omani sheep in order to establish some of the basic features of these parasites at two sites in central Oman.
Blood samples and ticks were collected from 231 animals. Hematological indices including PCV, RBCs count, Hb, and WBCs count were determined. In addition, specimens were collected from 11 sheep suspected to have died of theileriosis. Microscopy and PCR-RFLP of the 18S r-RNA were used for identification of *Theileria* species.
T. ovis has been identified as the main parasite in Oman, while *T. lestoquardi* and *T. annulata* existed at low prevalence, often as mixed species infection. *T.ovis* was found to cause significant blood pathology (anaemia and lowered PCV), and in a high proportion of cases (>80%), it was associated with the presence of schizont stage in the blood. However, *T. ovis* is conventionally considered as avirulent and does not cause significant proliferation of infected leukocytes (non-transforming) but rapidly differentiates to merozoites. In contrast, *T. lestoquardi* was not associated with significant pathology. Based on sequence analysis of the 18S r-RNA gene *T. lestoquardi* was found to be distinct, but closely related to *T. lestoquardi* from Iran and Sudan. Many spices of ticks of the genus *Hyalomma* were detected, and at least 20% of the examined sheep were infested.
Our data demonstrated that theileriosis is highly prevalent in Oman; and raised questions that warrant further work; first, has *T. ovis* evolved to be both pathogenic and host cell transforming or could the schizonts indicate a novel *Theileria* species that is not the source of the PCR amplicon? Second, do *T. lestoquardi* parasites in Oman belong to an avirulent sub-type of this species with a reduced pathogenicity to sheep in the region.

A molecular survey of *Eimeria* species occurrence in African and Asian poultry

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Protozoan parasites of the genus *Eimeria* can cause a severe enteritis called coccidiosis that affects many livestock species, most notably poultry. Recently, cost effective recombinant anticoccidial vaccines have become a more realistic prospect following the identification of new immunoprotective antigens. To predict the likely efficacy and longevity of such vaccines in the field it is important to define the occurrence of each *Eimeria* species and the prevalence of naturally occurring genetic (antigenic) diversity. To date most subunit vaccine development has been based on established laboratory strains from Europe and North America and little is known about genetic diversity of *Eimeria* species in other regions. In response to this deficit fresh faecal samples were collected from commercial broiler and layer farms within Egypt, Ghana, India, Libya, Nigeria, Tanzania, Uganda and Zambia. Field samples were screened for the presence or absence of each of the seven *Eimeria* species that cause coccidiosis in the chicken, as well as the three cryptic genotypes identified from surveys of Australian poultry as 'operational taxonomic units' (OTUs) X-Z, using species-specific PCR and internal transcribed spacer (ITS) sequencing. *Eimeria* were found to be widespread within African and Indian poultry and were present on more than 90% of farms sampled. Species complexity was comparable to that of Europe and North America, with all seven recognised *Eimeria* species detected and many farms concurrently infected by multiple species. Sequences consistent with the presence of OTUs X and Z but not Y were identified for the first time outside of Australia. The detection of genetically variant parasite strains, with unknown specificities and pathogenicities, in regions where they have not previously been described may compromise existing anticoccidial control strategies indicating elevated risk to food security.

Comparison of the structure and frequency of simple tandem repeats (STRs) between the genome of bovine apicomplexan pathogens, *Babesia bovis* and *Theileria annulata*

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Babesia bovis and *Theileria annulata* are the main etiological agents of bovine babesiosis and tropical theileriosis, respectively, affecting cattle health in many tropical and subtropical regions around the world. Both parasites are closely related and are referred to as piroplasmids. They show a similar life cycle, are transmitted by ticks to their cattle host and both own a genome of about 8 Mb size which is arranged in four chromosomes. Developing a multi-locus typing system we incidentally found great differences of simple tandem repeat (STRs) frequencies and structure in their genomes. Remarkably, the *T. annulata* genome contained a 7 time higher frequency of STRs compared to that of *B. bovis*. To find the basis of this surprising difference the STR frequency of coding, intronic and intergenic sub-regions was also assessed and compared. In *T. annulata*, 50% of intronic and intergenic regions are formed by STRs, while only 15% of coding regions contain repetitive sequences. In contrast, intergenic regions of *B. bovis*, are composed by 4.5% of STRs, while only 2% of intronic and coding regions are represented by STRs. Thus, the difference in STR frequencies in the whole genome of both species is reflected in each of the three genomic subregions. However, in both parasites, a significant lower proportion of STRs was observed in coding as compared to intergenic and/or intronic regions. As expected, a negative selection of STRs with a period size different from three or multiples of three was observed in coding regions, as insertions/deletions would likely result in non-functional gene products due to frame shifts. Remarkably, *T. annulata* showed a significantly higher number of STRs of small period size with respect to *B. bovis* as compared to other size classes of STRs. The possible biological function and molecular mechanisms leading to these differences are discussed.

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Detection of *Cryptosporidium* (Apicomplexa, Sporozoa) in rodents, farm animals and wild birds in Gobustan

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Previously, it has been found that in Azerbaijan *Cryptosporidium* is widespread in farm animals and some wild animals. In this study, we aimed to find out whether there is a circulation of *Cryptosporidium* between farm and wild animals. For this purpose, we studied rodents, wild birds and farm animals in the modified ecosystem of Gobustan Plateau. This ecosystem has been significantly transformed by human activity; farm animals and a variety of wild animals live in a limited area and they feed on a common vegetation.

We investigated fecal samples of two species of rodents: widespread red-tailed gerbil (*Pallasiomys erythrourus*) and Mountain Asian jerboa (*Allactaga williamsi*), as well as birds Isabelline wheatear (*Oenanthe isabellina*), who nest in rodents' burrows. In farm animals, we examined isolated faecal samples of cattle and sheep. The detection of *Cryptosporidium* oocysts was conducted with the use of light microscopy (by Ziehl-Neelsen).

Oocysts were found in 93 (51.9%) of the 179 tested feces samples of cattle; in 48 (33.6%) of 143 samples taken from sheep, in 22 (48.9%) of 45 samples from red-tailed gerbil in 1 (33.3%) of 3 Mountain Asian jerboa, and 15 (18.6%) of 86 Isabelline wheatear samples.

We found that the morphometric characteristics of *Cryptosporidium* oocysts detected in both wild and farm animals match: all oocysts have spherical dimensions 5.0x4.5 µm. However, in rodents, larger oocysts (size 7.0x5.0 µm) were also found. Based on the size, we believe that the *Cryptosporidium* oocysts which we found in both wild and domestic animals belong *Cryptosporidium parvum*, and large *Cryptosporidium* oocysts which we found in rodents only, belong *Cryptosporidium muris*.

We believe that the method of microscopy has not lost its significance, as it is easily available, cheap, and still remains the most widely used method of detection of *Cryptosporidium* oocysts. But the application of new immunological molecular methods will help to establish whether there is a general circulation of *Cryptosporidium* species between wild and farm animals. Perhaps the use of genetic and molecular methods will allow to clarify the species composition of *Cryptosporidium* of animals in Azerbaijan. Our research is continued in this direction.

Molecular characterization of *Cryptosporidium* isolates from calves with diarrhea in Konya Province

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This study was carried out to determine the prevalence and molecular characterization of *Cryptosporidium* species in totally 250 faecal samples collected from up to 2 months calves with diarrhea in Konya province and supported by Erciyes University Scientific Research Fund with the project number TSA-11-3748. Modified-Ziehl-Neelsen Staining technique was used to investigate *Cryptosporidium* oocysts in fecal samples. After the genomic DNA extraction from the fecal samples, TaqMan probe based real time PCR analyses were carried out with *Cryptosporidium* spp. and *C. parvum* specific primer pairs which amplify different size regions of partial 18S rRNA and a polymorphic gene, respectively. Furthermore all samples were analyzed by Nested PCR for the amplification of small subunit (SSU) rRNA gene region. Totally 92 (47.4%), 104 (53.6%) and 84 (43.3%) out of examined samples were found positive by microscopic, Real Time PCR and Nested PCR analyses, respectively. All positive samples were identified as *C. parvum* by Real Time PCR species specific assays. The amplicons obtained by Nested PCR from the positive calves were further analyzed by RFLP using VspI, SspI and MboII restriction enzymes in order to determine the genotypes and mix infections. According to the RFLP results, all samples which exhibited reaction in Nested PCR showed specific DNA band profiles for *C. parvum* with all three restriction enzymes and no mix infections were determined. The phylogenetic analyzes of 18S rRNA gene region revealed that the obtained *C. parvum* isolates showed 100% identity with each other and 0.3% genetic distance with *C. parvum* isolates available in GenBank. Furthermore, only one zoonotic *C. parvum* subtype IIaA13G2R1 was determined according to the analysis of the GP60 gene region of the isolates. The Real Time PCR assay was found to be the most sensitive technique for identifying *Cryptosporidium* infections especially in animals with lower parasitemia.

Molecular identification of *Sarcocystis* spp. in foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) from Germany

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Canids are definitive hosts (DH) of several *Sarcocystis* spp., which affect a wide range of intermediate hosts (IH) worldwide. The aim of the present study was to identify the *Sarcocystis* spp. present in the small intestine from foxes and raccoon dogs captured in Brandenburg, Germany, using molecular tools. A total of 50 or 38 mucosal scrapings from foxes (F) or raccoon dogs (RD), respectively, were collected as part of an Echinococcosis surveillance program, frozen at -80°C for at least one week and conserved at -20°C. The samples were analyzed by sugar flotation and when sporocysts/oocysts were detected, an overnight sedimentation was performed. DNA was extracted from aliquots of the sediment and the flotation concentrate using a commercial kit. A PCR was conducted using primers targeting a fragment of the 18S rRNA gene (≈850 bp). Amplicons were purified and sequenced. Samples with inconclusive sequencing results were cloned into plasmids and ≥ 3 plasmids from each amplicon were sequenced. *Sarcocystis* spp. infection was detected in 38% (19/50) and 52.6% (20/38), and mixed infection was recorded in 6 and 4 samples, of F and RD samples, respectively.
Sequences from F samples showed ≥ 99% identity with *S. capracanis* (n=6), *S. gracilis* (n=5), *S. capreolicanis* (n=4), *S. miescheriana* (n=4), *Sarcocystis* spp. using birds as IH (n=3), *S. grueneri* (n=1), and *Cystoisospora* spp. (n=2). In addition, one sequence with 97% identity with *S. cruzi* was detected. Out of all RD samples, sequences with a ≥ 99% identity with *S. miescheriana* (n=7), *S. gracilis* (n=6), *Sarcocystis* spp. using birds as IH (n=5), *S. capreolicanis* (n=4), *S. capracanis* (n=1) and one sequence with 96% identity with *S. cruzi* were observed.
The prevalence of *Sarcocystis* spp. infection observed in this study using mucosal scrapings was higher than related studies performed by analyzing stool samples. The present study represents the first molecular approach to identify *Sarcocystis* spp. infections from mucosal scrapings. It showed the utility of this approach to identify potential DH of these species.

Notchless is a key regulator of *Toxoplasma gondii* growth and proliferation

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Toxoplasma gondii is an obligate intracellular parasite, which only replicates following completion of the host cell invasion process. The mechanism involved in the tight control of parasite replication is not known. Notchless (NLE) encodes a broadly conserved WD40-repeat-containing protein with an N-terminal Nle-domain previously shown to bind to the cytoplasmic domain of Notch and hence to modulate Notch signaling activity in *Drosophila*. NLE is also involved in a variety of developmental pathways in plants and animals despite the absence of Notch signaling. Work performed in yeast has demonstrated that NLE (Rsa4), as well as the other Nle-domain and WD40 repeat-containing protein YTM1, act as pre-ribosomal factors. Together with a dynein-related AAA-ATPase (Rea1 or Midasin), these nucleolar (YMT1) and nuclear (NLE) proteins participate in the maturation and export of the 60S ribosomal particles.
In *T. gondii*, NLE is cytosolic in extracellular parasites and locates to the nucleus as soon as parasite enters the host cell. Moreover TgNLE level fluctuates during the parasite cell cycle. A conditional depletion of TgNLE or the conditional stabilization of a dominant mutant form of TgYTM1 (unable to bind to Midasin and to dissociate form the pre-60S) fused the KFBP destabilization domain severely arrest parasite division. Polysomes profile analysis of these parasite mutants confirmed a blockade in the pre-60S maturation process and protein synthesis was shown to be stronglx reduced. The mechanisms governing nuclear translocation of TgNLE and fluctuation during cell cycle progression remain to be determined. However these data suggest that *T. gondii* could use ribosome biogenesis and protein synthesis as a way to control replication between the extracellular and intracellular phases.

Comparison of host cell invasion and proliferation among *Neospora caninum* isolates obtained from oocysts and from clinical cases of naturally infected dogs

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In a previous study we have shown that the *in vitro* invasion rate (IR) and tachyzoite yield (TY) are associated with the virulence phenotypes of *Neospora caninum* isolates of bovine origin. In addition, we recently observed marked differences in virulence, when canine isolates were compared in a pregnant BALB/c mouse model. In this study, we investigated whether invasion and proliferation capacities could be used as virulence-related *N. caninum* phenotypic traits. Of the isolates compared, four canine isolates obtained from oocysts (Nc-Ger2, Nc-Ger3, Nc-Ger-6, Nc-6 Arg) had shown a low-moderate virulence, and two further isolates obtained from dogs with neurological signs (Nc-Bahia, Nc-Liv) were highly virulent in mice. The IR for each isolate was determined by a plaque assay and the counting of immunofluorescence-labeled parasitophorous vacuoles at 3 days post-inoculation (p.i.). The TY was determined by the quantification of tachyzoites at 56 h p.i. by real-time PCR. Most of the canine isolates were found to have similar IR values under controlled invasion conditions for 4 h and 72 h p.i., indicating a limited time period for invasion similar to that observed for bovine isolates. The Nc-Ger3, Nc-Bahia, and Nc-Liv isolates showed a significantly higher IR and TY than the Nc-Ger2 and Nc-Ger6 isolates ($P < 0.0001$). A correlation was found between the IR_s and TY ($\rho > 0.885$, $P \leq 0.033$), as well as between the TY and both dam morbidity ($\rho = 0.8452$, $P \leq 0.033$) and pup mortality ($\rho > 0.8117$, $P \leq 0.058$) in pregnant mice. These results demonstrate the importance both the invasive and proliferative capacities have on the virulence of canine *N. caninum* isolates.

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Besnoitia besnoiti and *Toxoplasma gondii*: different strategies to interact with the microtubule cytoskeleleton of the host cell – different outcomes in invasion and replication assays

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Besnoitia besnoiti and *Toxoplasma gondii*, two closely related apicomplexan parasites, interact with the host cell microtubule cytoskeleton, during the first steps of host cell invasion and parasite replication, since host cell microtubules organized around the established parasitophorous vacuole are constantly observed. For *T.gondii*, but not for *B.besnoiti* this interaction implicates the recruitment of the host cell centrosome, the primary microtubule organizing center. When using as host cells RPE-1overexpressing TBCCD1, a protein involved in nucleus-centrosome connection, the centrosome recruiting efficiency by *T.gondii* decreases.

These differences found in host cell centrosome manipulation during invasion by *T.gondii* and *B.besnoiti* were addressed in invasion and replication assays. In the case of *T.gondii*, the replication rate in cells overexpressing TBCCD1 decreases, compared with WT control cells, which can be linked to a higher difficulty in recruiting the centrosome. In addition, in host cells where *tbccd1* gene was knockdown causing the displacement of the centrosome from the nucleus, no difference is found in the total number of invaded cells.

However, there is a diminished number of parasitophorous vacuoles *per* invaded cell, when compared to control cells. Interestingly, in *B.besnoiti*, which does not recruit the host cell centrosome, during invasion of either cells overexpressing TBCCD1 or TBCCD1 depleted, there is no alteration in the rate of replication, invasion (total number of invaded cells), or in the number of parasitophorous vacuoles *per* invaded cell.

Our results strongly support the idea that *T.gondii* host cell invasion and parasite establishment/development, involves the recruitment of the centrosome towards the parasitophorous vacuole, and that this recruitment is required for parasite replication. Clearly, *B.besnoiti* has developed a different strategy to invade and replicate in host cells that does not require centrosomes of the host cell nearby the parasitophorous vacuole.

These differences might reflect two distinct evolutionary invasion mechanisms used by the two parasites.

Besnoitia besnoiti infections up-regulate immunomodulatory molecules in primary bovine endothelial cells

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Bovine besnoitiosis is an important protozoan disease that is considered as emerging in the EU owing to its rapid spread within the last years. *In vivo*, acute stage proliferation of *B. besnoiti* tachyzoites mainly takes place in highly reactive endothelial cells. Upon activation, these cells are able to produce a broad panel of immunomodulatory molecules, such as adhesion molecules, chemokines and cytokines, thereby initiating early inflammatory immune responses. To our knowledge there are no data available on such immunomodulatory mechanisms in *B. besnoiti*-infected bovine endothelial cells so far.

In the present study primary bovine umbilical vein endothelial cells (BUVEC) were infected with *B. besnoiti* tachyzoites and the gene transcription of adhesion molecules (VCAM-1, ICAM-1, P- and E-selectin), chemokines (CXCL1, CXCL8, CCL2, CCL5), IL-6, GM-CSF, iNOS and COX-2 was analysed at different time points p. i. (6, 12, 24, 48 h) using quantitative real-time RT-PCR. Furthermore, adhesion of polymorphonuclear neutrophils (PMN) onto *B. besnoiti*-infected BUVEC monolayers was estimated in a parallel flow chamber applying physiological flow conditions.

B. besnoiti infection clearly led to BUVEC activation since the gene transcripts of all immunomodulatory molecules mentioned above were enhanced during *in vitro* infection when compared with non-infected controls. Overall, the highest up-regulation of transcription was observed at 12 h p. i. Enhanced adhesion molecule expression was confirmed by PMN adhesion assays. Thus, significantly increased PMN adhesion was measured on *B. besnoiti*-infected BUVEC monolayers 24 h p. i. It is worth noting, that parasite-triggered PMN adhesion was remarkably strong since almost equal reactions were induced by TNF-α stimulation or *B. besnoiti* infections.

The present study shows that *B. besnoiti* infections of primary bovine endothelial cells induce a cascade of proinflammatory reactions and triggers early innate immune responses which may be of relevance in the *in vivo* situation.

The *Rhipicephalus microplus* VDAC protein is upregulated in midgut cells during the infection with *Babesia bigemina*

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The study of the molecular mechanisms involved during the infection of *Rhipicephalus microplus* midgut cells by *Babesia bigemina* is extremely relevant and so far they are unknown. In a previous study we found a Voltage Dependent Anion Channel (VDAC) that might be participating during parasite invasion to the midgut cells. In this work we investigated the VDAC expression at mRNA and protein levels, also we studied the cellular VDAC localization in midgut cells in ticks infected with *B. bigemina* at different times post-repletion. *R. microplus* adult females were fed on a *B. bigemina*-infected bovine or on an uninfected one. Midgut cells were dissected and processed for *vdac* mRNA quantitation, protein analysis by western blot and VDAC localization by indirect inmmunofluorescence antibody test (IFAT) at 0, 6, 12, 24, 48 and 72 hours post-repletion. According to the RT-PCR results, *vdac* expression level was significant higher in infected ticks compared to uninfected ones reaching the highest value at 24h post-repletion, same results were obtained at the protein level as determined by WB. Interestingly, VDAC was localized in the plasmatic cell membrane by indirect immunofluorescence, with a higher fluorescence signal in infected ticks at 24h post-repletion. VDAC is known as a mitochondrial porin with multiple functions, although it has been found in plasma membranes in other organisms. This is the first report of *Rm*VDAC up-regulation and immune-localization in *R. microplus* midgut cells during the infection by *B. bigemina*. Further studies about the function of *Rm*VDAC during the infection may provide new insights into the molecular mechanisms between *B. bigemina* and its tick vector, and could lead as well to its use as an anti-tick and transmission-blocking vaccine candidate.

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Proteins produced by *Babesia ovis* during the infection process

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Babesia ovis is an intraerythrocytic parasite that is transmitted to small ruminants by ticks and can cause Babesiosis to the cattle. During the infection process, the parasites produce proteins that recognize the receptor of the erythrocytes to infect them. To study this stage of the infection, the parasites were grown in two different serum-free media cultures (PBS and VyM). Since both mediums enable the parasite to invade the host cell, is possible to establish a comparison in terms of produced proteins between these mediums and CM culture. After 2h, 18h and 24h of infection the proteins of the parasite were extracted and resolved on 1D SDS-page gel electrophoresis.

The results shown that similar protein profiles were obtained in all media, especially after 24h infection, and that the proteins with 14 kDa and 62kDa were common to all of them. Two other proteins (MW of 25kDa and 30kDa) were also detected in VyM and CM and respective replicas and being the later also detected in one replica of PBS.

To identify immunodominant proteins excreted by the parasite a Western blot analysis was performed using positive and negative sheep sera as the primary antibody. The results showed two proteins (MW around 25 and 30 kDa) produced by *Babesia* in PBS 18h after infection that reacted when the positive serum was added. The controls did not show the positive reaction.

2D electrophoresis of the proteins produced by *Babesia ovis* during infection

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Babesia ovis is a parasite that infects red blood cells (RBC) of sheep's. The disease caused by the parasite results in several economic losses to the livestock industry.
The establishment of *in vitro* cell culture allows the study of the infection process of the parasite with higher levels of parasitaemia than the ones found in infected animals. This permits the study of parameters that can be individually manipulated to monitor its overall effect on the parasite development. In this study three different culture media were used in order to study the proteins produced by the parasite. For each media, three different time-points were evaluated (2h, 18h and 24h).
PBS and VyM are two serum-free media that enable the parasite to enter the erythrocyte without completing their asexual stage, but in VyM, *Babesia* can complete its life cycle at least a couple of times in a slower way. The third media is complete medium (CM) culture, which is the normal medium used on *in vitro* culture.
The proteins were extracted and resolved on 1D SDS-Page gel electrophoresis and also on pH 3-10 2D electrophoresis.
The results show low expression of proteins in all the 3 media. After 2h and 18h of infection two proteins were expressed in almost all media and their respective replicates, corresponding to protein spots around 14 kDa and 62kDa of MW. In VyM two replicates have additional proteins around 28kDa and 55kDa. After, 24h of infection the VyM and CM media have similar protein profiles, but with a higher number of proteins expressed in CM medium, which is expected since in CM the parasites are able to complete its asexual phase. The 2D gel approach revealed the presence of several proteins isoforms. In future the spots will be digested and the proteins identified using MALDI-TOF/TOF apparatus.

Eimeria arloingi sporozoites induce "Neutrophil extracellular traps" as innate immune reaction

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Neutrophil extracellular traps (NETs) are a major effector mechanism of polymorphonuclear neutrophils (PMN) leading to extracellular entrapment and killing of invasive pathogens. NETs are released upon activation of PMN and consist of a DNA backbone decorated with granular contents, such as myeloperoxidase (MPO) and neutrophil elastase (NE). So far, no data are available on the role of NETs in innate immune responses against the goat apicomplexan parasite *Eimeria arloingi*. For the first time, we here demonstrate the relevance of this important innate defense mechanism in PMN of the species goat reacting with different *E. arloingi* stages. Thus, scanning and fluorescence microscopic analyses revealed fine networks of DNA fibrils with co-localized histones (H3), NE and MPO in extracellular trap-like structures released from caprine PMN upon confrontation with *E. arloingi* stages. Overall, NETs were induced by both, oocysts and sporozoites irrespective of their viability. As expected, treatments of sporozoite-confronted caprine PMN with DNase and diphenylene iodondium significantly reduced NET formation. In addition, NE enzymatic activity and the production of reactive oxygen species (ROS) were found enhanced in sporozoite-triggered PMN, whilst MPO activity was not influenced. Entrapments assays revealed a proportion of 72% of sporozoites being entrapped in NET structures, reducing the sporozoite's invasive potential for adequate host cells. These findings suggest NETosis as an important innate immune mechanism in the early defense of *E. arloingi* infections in goats.

Host cell cholesterol esterification blockage inhibits *Eimeria bovis* development *in vitro*

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Eimeria bovis is an obligate intracellular coccidian parasite that exhibits enormous proliferation during macromeront formation within endothelial host cells. We have recently shown that the parasite actively interferes with host cell cholesterol metabolism for successful replication. In this study we analyzed the role of host acyl-CoA: cholesterol acyltransferase (ACAT), an enzyme that mediates cholesterol esterification. Since cholesterylesters are mainly stored in host cell lipid bodies we furthermore investigated the influence of *E. bovis* infection on lipid body formation in endothelial host cells.
Our results showed that ACAT2 gene transcription was up-regulated in *E. bovis*-infected endothelial host cells. Furthermore, *E. bovis* infection induced strong accumulation of lipid droplets in both host cell cytosol and the parasitophorous vacuole. Quantitative assays performed at different time points of infection (2, 6, 10, 14, 18, 22, 26 d p.i.) revealed the highest lipid droplet accumulation to occur prior to merozoite maturation indicating the parasite's needs for cellular lipids for merozoite formation. Inhibition studies applying CI976 for ACAT blockage demonstrated a key role of this enzyme in successful parasite replication. Thus, treatment of infected cells with CI976 led to an arrest of parasite development irrespective of the time point of CI976 application. When applied from the first day of infection onwards, ACAT inhibition led to significantly decreased infection rates during parasite maturation. Furthermore, the sizes of developing meronts were significantly smaller in treated cells when compared to non-treated ones. In addition, CI976-treated cells released significantly less merozoites over time resulting in a blockage of up to 90 % of merozoite production in total.
These findings confirm that *E. bovis* actively interferes with the host cell cholesterol and lipid metabolism and that especially cholesterol esterification and lipid body formation are key processes for successful *E. bovis* proliferation *in vitro*.

Avian coccidia: intestinal terrorists but systemic saviours?

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Eimeria can cause the disease coccidiosis in all livestock, most notably poultry. While the direct impact of coccidiosis on animal production and welfare has long been recognised, the influence of infection on the enteric microbiota remains largely unknown with the possible exception of *Clostridium perfringens*. The relevance of a balanced microbiota to metabolic efficiency and protection against pathogen colonisation indicates a broader impact of eimerian infection, even in the absence of clinical disease.

Campylobacter, most famously associated with human infectious intestinal disease, has also been implicated as an infectious pathogen of poultry. Concerns relating to zoonotic potential as well as impact on economic food production and animal welfare now elevate the political and social relevance of this bacterium. 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 has revealed significant variation in the presence of concurrent *Eimeria tenella* infection. Intriguingly, parasite co-infection associates with an elevated *C. jejuni* load within the caecal lumen three and ten days post bacterial challenge but reduced colonisation of the liver and spleen. Thus, while faecal shedding of *C. jejuni* is at least temporarily increased by overlapping *E. tenella* infection, deep tissue bacterial contamination is decreased. Underlying mechanisms will be discussed including roles for mucus and innate immune responses.

Building on these studies the influence of eimerian infection on microbiome composition and pathogen colonisation of poultry may impact on the development of *Eimeria* as vaccine delivery vectors. Importantly, more *Campylobacter* outbreaks are now associated with consumption of undercooked poultry liver pâté or parfait than faecal contamination of meat surfaces. For *C. jejuni* the public health risk associated with contaminated chicken liver may promote use of live eimerian vaccines.

Effect of artemisinin on immune system in chickens: preliminary results

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We investigated the effect of artemisinin on immune system in broiler chickens following chronic oral intake by lymphocyte proliferation assay (mitogens: concavalin A, lipopolysaccharide, artemisinin), phagocytosis assay, evaluation of serum gamma-globulin (SGG) levels, and development of lymphoid organs (bursa of Fabricius, spleen, and thymus). The chickens received artemisinin (5, 50, and 500 ppm) in their diet from 18 to 46 day-old. The level of serum gamma-globulin was significantly higher in chickens in-feed treated with artemisinin than in untreated chickens. However, only in chickens treated with 5 and 50 ppm the level of SGG increased over time, while in chickens treated with 500 ppm decreased as in control group. The *in vitro* phagocytic activity was supressed by artemisinin only at 28 days after in-feed treatment with artemisinin, but without statistical significance comparing with control group. Interestingly, in variants without stimulation, and stimulation with lipopolysaccharide, in-feed treatment with 50, and 500 ppm artemisinin stimulated the *in vitro* phagocytic activity. As regarding the thymus weight we did not registered statistical significant difference among the groups, even chickens in-feed treated with 50, and 500 ppm artemisinin had a lower weight. Generally, the stimulation index of lymphocytes was higer in chickens treated with 50, and 500 ppm artemisinin and *in vitro* stimulated with concavalin A and lipopolysaccharide, but lower in case of *in vitro* stimulation with artemisinin. Over time, stimulation index decreased in all treated chickens, but greater in chickens treated with 50 ppm artemisinin, and *in vitro* stimulated with concavalin A and artemisinin, while in case of lipopolysaccharide increased. Also, the weight of bursa was significant lower in chickens treated with 50 ppm artemisinin, followed by chickens treated with 500 ppm artemisinin, comparing with untreated chickens. The spleen had a lower weight in chickens treated with 50, and 500 ppm artemisinin, but without any statistical significant difference. Artemisinin is relatively immunotolerant in broiler chickens, and further investigations are needed to make a final conclusion.

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Glycosylphosphatidylinositol metabolism is critical for the survival of bovine *Babesia* parasites

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Glycosylphosphatidylinositols (GPIs), formed by an inositol phospholipid attached to a chain of typically three mannose molecules, are abundantly present in several protozoan parasites, and participate in host-pathogen interactions, both as modulators of host immune responses, and as key molecules in the parasitic life cycle. We have focused on the GPI metabolism of *Babesia bovis* and *B. bigemina*, the main causative agents of bovine babesiosis, in the search for new therapeutic targets. *In silico* we identified the genes responsible for the GPI metabolic pathway in the fully annotated genome of *B. bovis*, by BLAST searches using the corresponding sequences of related eukaryotes. In the case of the non-annotated genome of *B. bigemina*, *in silico* search in genomic contigs revealed the DNA segments containing the genes of interest which were PCR-amplified and sequenced. Both parasites, *B. bovis* and *B. bigemina* contain eight enzymes (PIG-A, GPI-1, PIG-L, PIG-W, DPM-1, PIG-M, PIG-V, PIG-O) that are involved in the synthesis of GPIs in the endoplasmic reticulum, starting from phosphatidylinositol, N-acetylglucosamine, dolichol-phosphate and mannose-GDP. The corresponding enzymatic steps are common to the production of free GPIs and GPI protein anchors. Subsequently, GPI-8, and GAA-1 catalyze the attachment of the GPI anchor to a nascent protein. RT-PCR studies performed on three of the identified genes showed that they are transcribed in pathogenic and attenuated strains of *B. bovis* and *B. bigemina*. Noteworthy, mannosamine, a competitive inhibitor of GPI synthesis, hampered *in vitro* growth of merozoites of both parasites, in a dose-dependent manner. These results are consistent with a critical role for GPI-anchored proteins in the attachment to and/or invasion of erythrocytes, a critical event in *Babesia* sp. life cycle. In addition, they could be indicative for an essential role of free GPIs in this process.

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The rhoptry-associated-protein 1 family in the sheep parasite *Babesia* sp. Xinjiang: multiple copies of the gene that differ by a variable number of sequence repeated blocks

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Sheep babesiosis is distributed mainly in the tropical and subtropical areas, with several species described as the etiological agent: *Babesia ovis*, *B. motasi* and *B. crassa*. Recently, several *Babesia* isolates infecting sheep and goat have been described in China. They belong to two phylogenetic groups: the *B. motasi* group and the *Babesia* sp. Xinjiang group. In order to develop recombinant sub-unit vaccines to target *Babesia* infecting small ruminant, we focused on RAP-1 (Rhoptry-Associated-Protein 1) as it is known to be a protein involved in the red blood cell invasion process.
For *Babesia* sp. Xinjiang, the *rap-1* locus genes have been amplified using degenerated primers designed from conserved motifs. Seven different genes have been sequenced. For all of them, the 5' regions had identical sequences over 936 nt, the 3' regions differ at 28 positions over 147 nt defining two types of genes, called α and β. According to the gene, the 3' remaining part of the gene varies in length from 72 to 360 nt. This region consists in a succession of 36 nt blocks, which numbers vary from 2 to 10, explaining the size difference. Even if the nucleotide sequences vary, 6 block sequences encode the same stretch of amino acids. B epitope mapping performed on the protein sequences reveals that each of these blocks contains a putative B epitope, and that most of the predicted B-epitopes were located at the C-terminal end of the protein. Analysis of the transcription of these different genes has been performed by standard RT-PCR: the transcription of at least 5 of them, from the α and β types, has been demonstrated. According to these results, the N-terminal conserved part of the protein could be used as a component of a multiple recombinant vaccine.

This work was supported by the European project KBBE PIROVAC. Q.N. PhD is supported by the PIROVAC project and by the Region Pays de la Loire. J.M. is supported by the PIROVAC project.

Metabolic profiling of culture medium of human brain microvascular endothelial cells challanged with *Neospora caninum*

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Neospora caninum is a leading cause of parasitic abortion in cattle and neuromuscular disease in dogs. However, the molecular framework regulating neuro-pathogenesis of this pathogen is poorly understood. We have previously showed that *N. caninum* efficiently invades and alters the bioenergetics of human blood-brain-barrier endothelial cells. This study aimed to assess if the pathophysiological impact of *N. caninum* infection on cultured endothelial cells could be explained by changes in the nutritional needs of endothelial cells during the course of intracellular (i.c.) infection. Fluctuations of culture medium nutrients in response to growth of *N. caninum* were determined using three approaches. First, concentrations of several extracellular biochemical parameters of HBMEC culture medium were monitored in a time course as a function of infection using a Randox RX Imola clinical chemistry analyser. Second, extracellular metabolite changes in culture medium were monitored by using Raman spectroscopy (RS). Third, a targeted LC-MS/MS method was employed to quantify important metabolite groups like amino acids, organic acids, sugar phosphates, other phosphorylated metabolites and nucleotides in the medium of infected cell cultures and control cultures at 24 pi. Cell viability was determined by LDH assay and demonstrated that *N. caninum* caused cytotoxicity in endothelial cells in a dose-response manner. Beta hydroxybutyrate, pyruvate, ATP, total protein (tp), non-esterified fatty acids (NEFA), and triglycerides were significantly different in infected cells compared with controls. RS and metabolite profiling using LC-MS/MS revealed alteration in cerian metabolites. The changing pattern of many metabolites reflects altered metabolism of the cultured cells in response to infection. These alterations seem to reflect the internal metabolic state of the infected cell and may form an under-appreciated level of information exchange between host and parasite to coordinate infection.

To bind or not to bind – Study of *Isospora suis* coccidial development through *Toxoplasma*-specific reagents

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An *in vitro* cultivation system has recently been developed for *Isospora suis*, a sister taxon of *Toxoplasma*, providing access to all developmental stages. Additionally, the *I. suis* genome has been sampled using Illumina sequencing. Sexual stages of coccidia are difficult to access and consequentially are only poorly understood. *In vitro* access and the fact that *I. suis* stages are relatively large in size offer opportunities to investigate the development of micro- and macrogametocytes in a reproducible system.

As a first step for analysis of *I. suis* development, antibodies targeting tubulins and relevant *Toxoplasma* cell division proteins were tested for immunofluorescence staining competence. In parallel, *I. suis* genomic sequences were aligned with selected *T. gondii* coding sequences. Identified contigs of interest were further used for gene prediction and corresponding hypothetical proteins of *I. suis* were identified.

Antibodies against acetylated tubulin led to specific staining of conoids, spindle poles and flagella. Together with staining of the kinetochore marker Nuf2 and of IMC1 and IMC3 it was possible to investigate nuclear division and budding dynamics of all meront types and of microgamonts from early nuclear division through to fully developed microgametes. However, the majority of antibodies did not stain specifically. Negative results were not always correlated with the lack of homologous hypothetical proteins in *I. suis*. In the absence of antibodies specific for proteins involved in the sexual development of *Toxoplasma*, and given inadequate amino acid similarity between putative *I. suis* proteins and homologues from parasites such as *Plasmodium*, there is now a requirement for the production of *Isospora*-specific reagents for detailed investigation of sexual stages. The results achieved using existing antibodies promise new insights into coccidial sexual development with early microgamonts exhibiting particular spindle pole and kinetochore patterns and the early formation of flagella detectable by specific β-tubulin staining.

L-cysteine replaces microaerophilous culture conditions for the *in vitro* initiation of *Theileria equi*

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Fifty-one blood samples of carrier horses from *Theileria equi*-endemic localities in South Africa were subjected to *in vitro* culture initiation of *T. equi* parasites using two different culture methods. Cultures were initiated either in an oxygen-reduced gas mixture or in a 5% CO_2-in-air atmosphere in combination with L-cysteine-supplemented culture medium. Out of the 51 blood samples, 43 and 42 cultures, respectively, became culture-positive using the two methods. A possible mechanism for the observation is suggested.

Generation and characterization of monoclonal antibodies against wall forming bodies of *Eimeria* macrogametocytes

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The vesicle transport of so called wall forming bodies (WFB) is a crucial step for oocyst wall formation in *Eimeria* species. Oocysts are the important duration stages, which allow to overcome adverse times without host. To build an oocyst is a complex cell biological task. Precursor proteins have to be transported to the periphery of the cell, have to be processed and involved into the covering protection system of the oocyst wall. Thus, preliminary steps like WFB formation and transportation are essential for a stage conversion which allows the parasites to survive in an untypical environment, outside the host cell.

To follow the cellbiological tracks of WFBs, two monoclonal antibodies (A1G8 and A6G7) were produced against gametocytes of *E. nieschulzi* and characterized. These antibodies are powerful tools for biochemical, microscopical and electron microscopical characterization of the oocyst wall forming process. The monoclonal antibodies A1G8 and A6G7 identified macrogametocyte proteins in the wall forming bodies of type II by indirect immunofluorescence tests. On western blots of purified gametocytes of *E. nieschulzi* seperated by SDS-PAGE, A1G8 recognized several bands between ~13 and ~60 kDa and A6G7 reacted with one prominent band of ~60 kDa under reducing conditions. Immunoelectron microscopy will be done.

The production of monoclonal antibodies is a current technique for identification and characterization of new gametocyte proteins and can shed more light on the oocyst wall formation process.

Endogenous development of the turkey coccidium *Eimeria meleagridis* Tyzzer, 1929

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The life cycle study was performed with the turkey coccidium *Eimeria meleagridis* Tyzzer, 1929. Turkey poults were inoculated with graded doses of oocysts and samples from the intestine were taken at 16-hour intervals. The samples were processed for histology and transmission electron microscopy. The coccidium formed three asexual generations followed by gamogony. All endogenous stages parasitized in the epithelial cells at the tips and walls of the intestinal villi. The first merogony that gave rise to a large number of merozoites (up to 60 or more) was seen in the ileum, while the remaining stages developed in the caeca. The second and third generation meronts were smaller than those of the first generation and were formed by up to 10 or 11 merozoites. The first oocysts were seen 128 hours after inoculation.

NOT PRESENTED

Long term viability of cryopreserved *Eimeria meleagridis* sporocysts

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Coccidiosis is a disease of many different animals caused by protozoan parasites of the genus *Eimeria* which invade and develop in the epithelial cells of the intestine.
Eimerian parasites are obligate parasites which can only reproduce within their host. Early workers involved in coccidiosis research realised that it was important to use "standard" strains which were obtained by isolating a single oocyst and passaging in the host to obtain a strain which could then be used for laboratory studies.
However, these strains could only remain viable for about a year when stored at $4^{O}C$, with the infectivity steadily declining over this period before they need to be repassaged in the host.
The passage of parasites in their host is both an expensive and ethical process, and the first indications that it was possible to cryopreserve coccidia were published by Kouwenhoven (1967) who froze sporocysts at $-79^{O}C$, and then Doran and Vetterling (1968), and Norton, Pout & Joyner (1968) who reported methods for preserving sporozoites in liquid nitrogen ($-196^{O}C$).
Researchers at the Central Veterinary Laboratory, Weybridge, UK (Now Animal Health and Veterinary Laboratories Agency) were extensively involved in coccidiosis research with *Eimeria* species from a number of hosts, including chickens, turkeys and game birds, and they developed methods for cryopreserving sporocysts rather then sporozoites (Norton & Joyner, 1968). The advantage in freezing sporocysts rather than sporozoites was that the parasite-stages could be recovered from liquid nitrogen and inoculated into the host animal via the oral route. This was important, because sporozoites could only be introduced into animals by direct inoculation into the intestinal tract, and this involved surgery.
The paper by and Norton & Joyner (1968) describing methods for the cryopreservation of sporocysts of coccidia from the chicken, *Eimeria acervulina, E. maxima*, and *E. tenella*, have been followed at this laboratory ever since with very few modifications.
This poster reports on the viability of sporocysts of *E. meleagridis* from the turkey (*Meleagris gallopavo*) after forty years in liquid nitrogen. The first author (RNM) was involved in all of this work, from freezing to infecting turkey poults forty years later.

Improved technologies to study *Toxoplasma gondii*

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We recently adapted the conditional recombination system based on the dimerization of Cre-recombinase (DiCre) to be used in *Toxoplasma gondii*. This inducible system allows a rapid and efficient excision of any piece of genomic DNA flanked by LoxP sites (floxed). This can be used for different approaches in the generation of conditional mutants in *T. gondii*: Knock-Out mutants (GeneSwap strategy) or mRNA degradation (Knock-down strategy).

The GeneSwap allows complete removal of the GOI (Gene of interest) and activates YFP-expression when a LoxP site at the 5'UTR of our GOI and a second LoxP site after the stop codon are introduced.

The Knock-down vector leads to endogenous tagging of the GOI with a HA tag and will allow localisation and biochemical analysis of the GOI. After induction of Cre recombination, degradation sequences are placed directly adjacent to the STOP codon of the GOI mRNA causing its degradation.

We generated a panel of GeneSwap or Knock-down targeting vectors to modify endogenous loci with LoxP sites with/without tags or reporter genes through homologous recombination. For alternative strategies we generated "indicator" strains to speed up and simplify the preparation of targeting constructs for homologous recombination: The current repertoire of technologies does not allow an easy and reliable generation of conditional mutants at high-throughput. Therefore, our future plans are to incorporate knock-out and knock-down approaches with recombination mediated genetic engineering to manipulate the *T. gondii* genome.

Differences in protein expression and similar antigenic profiles between *Besnoitia besnoiti* and *Besnoitia tarandi*

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Besnoitia besnoiti and *Besnoitia tarandi* are two cyst forming apicomplexan parasites that belong to the genus *Besnoitia*. *B. besnoiti* is the causative agent of bovine besnoitiosis, an emerging disease in Europe. Cattle are the intermediate host of this parasite, where both the tachyzoite and bradyzoite parasite stages develop and are responsible for the acute anasarca and the chronic scleroderma stage of the disease, respectively. On the other hand, wild ruminants act as intermediate hosts for *B. tarandi* which causes similar clinical signs as those originated by *B. besnoiti*. Many biological and epidemiological aspects inherent to these *Besnoitia* species remain unknown. Previous studies demonstrated strong uniformity at molecular level between both *Besnoitia* spp. since they conserve identical rDNA gene sequences. Moreover the existence of strong serological cross-reactions between them was recently demonstrated. In this sense, a difference gel electrophoresis (DIGE) approach and mass spectrometry (MS) analysis were used for a better characterization of both species including proteome description and detection of protein abundance differences between *B. besnoiti* (Bb-Spain 1 isolate, obtained from a naturally infected cow) and *B. tarandi* (isolated from a naturally infected reindeer in Finland) in tachyzoite extracts. Immunoproteomes were also compared by 2-DE immunoblot using pH 3-10 NL strips and polyclonal sera from experimentally infected rabbits. From approximately 1,400 spots visualized in 2DE-gels, 28 and 29 spots were differentially expressed in *B. besnoiti* and *B. tarandi* tachyzoites, respectively (average ratio±1·5, P<0·05 in t-test). Furthermore, 32 differentially expressed spots were selected and analyzed by MALDI-TOF/MS. As a result, 6 up-regulated *B. besnoiti* proteins (LDH; HSP90; purine nucleoside phosphorylase and 3 hypothetical proteins) and 6 up-regulated *B. tarandi* proteins (G3PDH; LDH; PDI; mRNA decapping protein and 2 hypothetical proteins) were identified. These results show marked differences in protein expression profiles between both species. In contrast, both *Besnoitia* species showed similar antigen profiles, corroborating the difficulty in elucidating epidemiological gaps by current serological assays. The present study evidences the need of sequencing *B. besnoiti* genome for complementary studies of DNA and protein characterization in genus *Besnoitia*.

Work supported by the Spanish Ministry of Economy and Competitiveness (M.I.N.E.C.O.; grant number: AGL-2010-20561/GAN). P. García Lunar fellowship is from Complutense Univ. of Madrid. DIGE experiment was carried out in the Proteomics Facility UCM-PCM - ProteoRed-ISCIII network.

Qualitative phospho-proteome analysis of *Theileria annulata* schizonts in infected bovine macrophages during the cell cycle

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The survival of the *Theileria annulata* schizont within the bovine leucocyte is ensured by the modulation of host cell signalling by the parasite, and a dramatic increase in kinase activity in the transformed cell has been well reported. Quickly after invasion of the sporozoite into the host cell, the parasite escapes the parasitophorus vacuole and interacts with the microtubule network. The parasite maintains its position within the continuously dividing host cell by closely interacting with the host cell mitotic apparatus, and recent work from our lab has suggested that the timely phosphorylation of the parasite surface may be required for these interactions. The interaction of host cell EB1 and Plk1 with the schizont surface has been described in detail by our lab. Both interactions were found to be negatively regulated by the activity of host cell Cdk1, and the schizont membrane protein p104, the binding partner of EB1, was reported to be phosphorylated in a cell cycle dependent manner. Plk1 activity was found to be crucial for the interaction of the schizont with the central spindle and the faithful segregation of the parasite during host cell mitosis. However no Plk1 substrates on the parasites surface have yet been identified. In fact, very little has been described regarding the phosphorylation of the parasite surface. We are now analysing phosphorylation events at the *T. annulata* schizont, and are focusing in particular on the difference of pT-, pTP- and pS-sites at the parasite surface. Based on microscopic anaylsis with specific antibodies we report that the parasite is phosphorylated differently throughout the cell cycle. Western blot analysis of schizont lysates obtained from cells blocked in S-Phase or mitosis were also performed, revealing cell cycle dependent differences in schizont phosphorylation patterns. We now intend to further analyse the *T. annulata* schizont phospho-proteome using mass spectrometry.

Drugs and drug targets in apicomplexan parasites: *Neospora*, *Toxoplasma*, *Theileria*

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Neospora caninum, Toxoplasma gondii and *Theileria annulata* are all intracellular apicomplexan parasites. *N. caninum* and the closely related *T. gondii* are responsible for reproductive failures in several ruminant species, and in the case of *Toxoplasma*, also humans. *Theileria annulata* and *T. parva* cause tropical theileriosis and East Coast fever in cattle, respectively. Although all these diseases represent major economic and veterinary public health concerns, the chemotherapeutical options available for anti-parasitic treatments are rather limited. Chemotherapy for the treatment of *N. caninum* infections has not been intensively studied and only few drugs have been used *in vivo*, although several compounds have been successfully tested *in vitro*. The standard treatment for human toxoplasmosis is based on drugs including spiramycin, sulfonamide and pyrimethamin, which can have severe side effects. Another issue is the lack of treatment for the elimination of tissue cysts. Tropical theileriosis and East Coast fever can be treated with buparvaquone and tetracycline, but resistance of *T. annulata* against buparvaquone has recently been reported, and tetracycline treatment alone is often not sufficient to cure the animal. Anti-parasitic compounds such as Artemisinin-derivatives are well established for the treatment of malaria and have been shown to exhibit pronounced anti-*Toxoplasma* activity and to affect *Neospora* development *in vitro* and *in vivo*. Related compounds have been assessed *in vitro* against *Neospora* and preliminary experiments have been performed to test the efficacy of those compounds against *Theileria* infected cells. On the other hand, recently developed inhibitors of calcium dependent kinases (CDPKs), which have been shown to impair *T. gondii* and *N. caninum* proliferation, exhibited no activity against *Theileria*-infected cells.

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