



## Analyzing Plasmodium falciparum erythrocyte membrane protein 1 gene expression by a next generation sequencing based method

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# JOINT SPRING SYMPOSIUM 2013

Danish Society for Parasitology and  
Danish Society for Tropical Medicine & International Health

## *The nature of parasitism*



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Friday 15<sup>th</sup> March, 2013, 8:30-16:00  
Faculty of Life Sciences, Lecture room 1-01 (Festauditoriet),  
Bülowsvej 17, 1870 Frederiksberg C

# ORAL PRESENTATIONS – KEY NOTES

## **The nature of parasitism**

Jørgen Kurtzhals

University of Copenhagen and Rigshospitalet

Parasites are organisms that for a part or the duration of their life depend upon colonization of a host. Parasitology is usually confined to helminths, protozoa, and arthropods but viruses, bacteria, and fungi commonly show parasitic properties. In my presentation, rather than focussing on specific classes of organisms, I wish to discuss the difference between virulent and parasitic organisms. Although many parasites are or can become virulent, this understanding of parasitism may help understand regulation at various levels.

The presentation will be restricted to three types of organisms. First, I will show how the interaction between host response and *Leishmania* spp. affect the clinical presentation and transmission of the infection. The study of this interaction led to the discovery of the Th1-Th2 paradigm of CD4+ lymphocytes and resulted in discovery of host factors regulating intracellular parasites. Secondly, I will discuss how bacteria are able to colonise the intestine without eliciting an immune response despite the immunologically active environment in the gut. This appears to be achieved by a Toll-like receptor pathway that differs from that activated by pathogen-associated molecular patterns (PAMPs) on virulent microorganisms. The term symbiont-associated molecular patterns (SAMPs) has been proposed to describe organisms that use the modified Toll-like receptor activation. Finally, I will discuss the multiple levels of parasitism seen in giant viruses that can both act as parasites of amoeba and as hosts of virophages. By studying these and a range of closely related virulent vs. non-virulent microorganisms it has been proposed that virulence may often be a result of genome reduction associated with loss of regulation.

## **Is *Plasmodium falciparum* a well-adapted parasite?**

Joseph Smith

Seattle Biomedical Research Institute, Seattle, WA, USA

Of the four major *Plasmodium* species that infect humans, *P. falciparum* is by far the deadliest. The increased virulence is associated with cytoadhesion of *P. falciparum* infected erythrocytes in blood microvessels mediated by *var* genes/PfEMP1 proteins. Whereas it was originally hypothesized that *P. falciparum* may be a more deadly parasite because it was a recent acquisition of human's agricultural life-style (~6000 yrs), recent phylogenetic analysis indicates the *P. falciparum* and human relationship is ancient. My lab is interested in the evolutionary selective pressures on the PfEMP1 protein family and the identification of PfEMP1 proteins that cause severe disease. We wish to understand how the parasite uses the cytoadhesion phenotype to favor survival and transmission and why the parasite retains deadly binding traits in its PfEMP1 repertoire ("cerebral malaria paradox"). I will discuss how we are applying repertoire-wide approaches to characterize adhesive protein functions in the PfEMP1 family and in vitro models of infected erythrocyte sequestration in different blood vessels. This analysis has demonstrated that PfEMP1 binding properties are remarkably predictive, despite extensive sequence diversity in the family. It has also uncovered an unusual subset of PfEMP1 variants (DC8 subset), which have exceptional binding activity for brain and non-brain sites and are associated severe malaria.

## **Ecology of Nature's Criminals: How to master a life with multiple hosts**

Brian Lund Fredensborg

Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen

A multitude of parasites need at least two hosts to complete their life cycle indicating the success of complex life cycles among parasites. This may seem counter intuitive given the minute chance of successful transmission between hosts and the diversity of environments that different parasite life cycle stages need to be adapted to. Thus, parasites with complex life cycles must be able to inhabit taxonomically unrelated hosts and cope with different physical habitats. Most importantly, parasites with complex life cycles must evolve means to get from one host to the next. This presentation provides a brief introduction to the evolution of complex life cycles, and gives examples of selection pressures acting on parasite life cycle stages and the intriguing array of adaptations that parasites use to transmit between hosts. Understanding those adaptations is important to gain more knowledge on parasite biology and to understand the evolutionary arms race between hosts and parasites. However, research on parasite adaptations that increase transmission also has very practical applications on control strategies of disease-causing parasites. Finally, the consequences of parasite adaptations on free-living animal communities will be discussed as will the potential applications of using parasites with complex life cycles as biological indicators.

## ORAL PRESENTATIONS

### **Fluctuations in prevalence of *Taenia solium* cysticercosis in pigs in Mbeya Region, Tanzania**

Uffe C Braae<sup>1</sup>, Erick V G Komba<sup>2</sup>, Stig M Thamsborg<sup>1</sup>, Helena Mejer<sup>1</sup> and Maria V Johansen<sup>1</sup>

<sup>1</sup>University of Copenhagen; <sup>2</sup>Sokoine University of Agriculture, Tanzania

*Taenia solium taeniosis/cysticercosis* is a serious agricultural and public health problem in Tanzania. This study aimed at describing the fluctuations in porcine cysticercosis prevalence in an endemic area. Three cross-sectional surveys were carried out in Mbeya Region, Tanzania, in November to December 2007, March to April 2012, and October to November 2012, respectively. In the first survey 300 farmers in 30 villages were visited and two of their pigs randomly sampled. In the following two surveys census sampling was used in 22 villages attempting to include all non-pregnant pigs more than 2-month old. For all surveys jugular vein blood was collected and analysed for cysticercosis antigen using Ag-ELISA. In the first survey 600 serum samples were collected. In each of the following surveys approximately 800 serum samples were collected. The first survey revealed a cysticercosis prevalence of 31% (n=600, CI=27-35%), in the second survey the prevalence had significantly dropped to 15% (n=822, CI=13-18%), and in the 6-month follow-up the prevalence had increased to 24% (n=812, CI=21-27%). Explanations for the observed fluctuations can only be speculated. However, in 2011 the study area suffered a decimating outbreak of African swine fever (ASF). Overlapping risk factors for ASF and porcine cysticercosis support the hypothesis that ASF may reduce cysticercosis prevalence. Also, as the Ag-ELISA assay used is not species specific, variation in transmission of other *Taenia* species could influence cysticercosis prevalence when measured by Ag-ELISA. Finally, seasonal variation in porcine cysticercosis could exist because of different seasonal production systems. The fluctuations are in contradiction with the theoretical model which predicts a stable equilibrium. Further studies are needed to determine whether the prevalence of cysticercosis has an endemic equilibrium, or in fact go through fluctuations with or without the presence of the factors described in this study.

## **Detection of cysteine protease in *Taenia solium* - induced brain granulomas in naturally infected pigs**

Ernatus M Mkupasi<sup>1</sup>, Chummy Sikalizyo Sikasunge<sup>2</sup>, Helena Aminiel Ngowi<sup>3</sup>, Pall S. Leifsson<sup>1</sup> and Maria V Johansen<sup>1</sup>

<sup>1</sup>University of Copenhagen; <sup>2</sup>University of Zambia; <sup>3</sup>Sokoine University of Agriculture, Tanzania

In order to further characterise the immune response around the viable or degenerating *Taenia solium* cysticerci in the pig brain, the involvement of cysteine protease in the immune evasion was assessed. Brain tissues from 30 adult pigs naturally infected with *T. solium* cysticercosis were subjected to histopathology using haematoxylin and eosin stain, and immunohistochemistry using caspase-3 antibodies. Histopathological evaluation revealed lesions of stage I which was characterized by presence of viable parasite surrounded with minimal to moderate inflammatory cells and stage III characterized by the presence of a disintegrating parasite surrounded with high inflammatory cells. The results of immunohistochemistry indicated caspase-3 positive cells interspaced between inflammatory infiltrate mainly in stage I lesions, indicating the presence of cysteine protease. This result confirms the earlier hypothesis that cysteine protease may play a role in inducing immune evasion through apoptosis around viable *T. solium* cysticerci.

## **Assessment of the societal burden of *Taenia solium* cysticercosis in Angónia district, Mozambique**

Chiara Trevisan<sup>1</sup>, Nicolas Praet<sup>2</sup>, Alberto Pondja<sup>3</sup>, Yunus A Assane<sup>3</sup>, Pierre Dorny<sup>2</sup>, Pascal Magnussen<sup>1</sup> and Maria V Johansen<sup>1</sup>

<sup>1</sup>Section for Parasitology, Health and Development, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen; <sup>2</sup>Institute of Tropical Medicine, Antwerp, Belgium; <sup>3</sup>Faculty of Veterinary Medicine, Eduardo Mondlane University, Maputo, Mozambique

*Taenia solium* cysticercosis is a zoonosis of both public health and agricultural importance in many endemic low-income countries. Neurocysticercosis may cause severe neurological disorders, the most common being epilepsy and headache. The Angónia district of Mozambique is a high endemic area for *T. solium* cysticercosis.

This study estimates the societal burden it has on the livelihood of the small rural society.

All data were compiled in the software for statistical analysis 'R version 2.15.1'. To estimate the Disability Adjusted Life Years (DALYs) lost due to NCC-associated epilepsy and headache a DALY calculator was used. To estimate the total costs a cost analysis model was used.

Based on a prevalence of epilepsy of 15.6% and of headache of 30.9% the number of people with NCC-associated epilepsy was estimated at 16,452 (95% CR, 14,553-18,462) and 2,662 (95% CR, 1,774 - 3,738) were the people with NCC-associated headaches. The number of adult pigs diagnosed with cysticercosis was estimated at 7,129 (95% CR, 6,401-7,879), which corresponded to 35% of the total adult pig population. The estimated average number of DALYs lost due to NCC-associated epilepsy and headache was 12.1 (95% CR, 7.0-19.1) per thousand persons per year. The total annual costs due to *T. solium* cysticercosis were estimated at 1,282,777 euro (95% CR, 770,775 - 1,910,569). The annual monetary burden per case of NCC-associated epilepsy amounted at 51.0 euro (95% CR, 29.00 - 76.50).

The DALY results of this study seem very high compared to other studies that estimated the health burden of the disease in other parts of the world. The cost estimates in Angónia district were lower than that of other studies carried out before.

The results show that *T. solium* cysticercosis is a serious public health and agricultural threat for Angónia district, Mozambique. It affects the livelihood of the rural society by reducing significantly its health and economy.



## **Analyzing *Plasmodium falciparum* erythrocyte membrane protein 1 gene expression by a next generation sequencing based method**

Jakob S Jespersen<sup>1</sup>, Bent Petersen<sup>2</sup>, Andaine Seguin-Orlando<sup>3</sup>, Eske Willerslev<sup>3</sup>, M Thomas P Gilbert<sup>3</sup>, John Lusingu<sup>1,4</sup>, Thor G Theander<sup>1</sup> and Thomas Lavstsen<sup>1</sup>

<sup>1</sup>Centre for Medical Parasitology, University of Copenhagen and Rigshospitalet; <sup>2</sup>Centre for Biological Sequence Analysis, DTU; <sup>3</sup>Centre for GeoGenetics, University of Copenhagen; <sup>4</sup>National Institute of Medical Research, Tanzania

*Plasmodium falciparum* is responsible for most cases of severe malaria and causes >1 million deaths every year. The particular virulence of this *Plasmodium* species is highly associated with the expression of certain members of the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) family, encoded by ~60 highly variable 'var' genes per haploid genome. PfEMP1 is exported to the surface of infected erythrocytes and is thought to be fundamental to immune evasion by adhesion to host and parasite factors. The highly variable nature has constituted a roadblock in var expression studies aimed at identifying PfEMP1 features associated with high virulence. Here we present the first effective method for sequence analysis of var genes expressed in field samples: a sequential PCR and next generation sequencing based technique applied on expressed var sequence tags and subsequently on long range PCR amplicons of the expressed vars. The results obtained with this method supports quantitative PCR data showing PfEMP1 of the group A and domain cassettes 8 and 13 types being expressed at particularly high levels in severe childhood malaria.

## **Antibodies to the C-terminal domains of *Plasmodium falciparum* erythrocyte membrane protein 1 may contribute to anti-rosetting immunity to malaria**

Liz Stevenson, Lea Barfod and Lars Hviid

Centre for Medical Parasitology, University of Copenhagen and Rigshospitalet

Rosetting, which is the binding of uninfected erythrocytes to a central *P. falciparum*-infected erythrocyte (IE), is an adhesion phenotype that has been associated with the pathogenesis of severe malaria, and rosette-inhibition antibodies are associated with clinical protection. Rosetting is mediated by the N-terminal head structure of particular variants of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) antigens on the IE surface. The C-terminal domains of some rosetting PfEMP1 proteins can bind IgM non-specifically, and this binding of IgM appears to augment rosetting.

Here, we used a particular PfEMP1 protein (HB3var06), which mediates rosetting and binds IgM non-specifically, to study the importance of the individual domains for rosetting. To this end, we produced recombinant proteins representing individual domains of HB3var06, domain-pairs, and full-length HB3var06. The constructs were used to immunize rats, and the ability of the anti-sera to inhibit rosetting in HB3var06-expressing parasites was tested.

Antisera to the N-terminal head structure of HB3var06 (NTS-DBLalpha and NTS-DBLalpha-CIDRdelta) completely inhibited rosette formation. However, other domain-specific antisera, including antisera to the two C-terminal DBLepsilon domains, also substantially inhibited rosette formation. Because of their C-terminal location, these latter domains are unlikely to be involved in rosetting directly. However, they sit on either side of the DBLzeta domain that appears to be involved in the binding of non-specific IgM to HB3var06.

On this basis we speculate that antibodies to the C-terminal part of IgM-binding PfEMP1 proteins involved in rosetting may contribute to protective, rosette-disrupting immunity to *P. falciparum* malaria by interfering with the IgM-binding that can stabilize or strengthen PfEMP1-mediated rosetting.

## **Inhibiting vascular endothelial growth factor signaling in *Plasmodium falciparum* attenuates its growth in vitro**

Casper Hempel, Nils Hoyer, Trine Staalsø and Jørgen AL Kurtzhals

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Host factors secreted during a malaria infection may either be detrimental or stimulating for parasite growth. There is evidence for vascular endothelial growth factor (VEGF) being able to stimulate intraerythrocytic growth of *Plasmodium falciparum*. Also, parasitized red blood cells accumulate VEGF in a malaria infection, but the cause of this uptake is not yet understood. Here, we demonstrate that growth during in vitro culture of *P. falciparum* is dose-dependently reduced when administering the specific VEGF receptor 2 (VEGFR-2) inhibitor SU5416. Growth was not inhibited when an anti-VEGF antibody, bevacizumab, was administered. The studies showed that VEGF accumulated intracellularly, particularly in schizonts. Supplementing the medium with both human and murine VEGF increased intracellular VEGF accumulation. Although SU5416 reduced growth rate, it did not reduce intracellular VEGF accumulation. Thus, the antiparasitic effect of VEGFR-2 inhibition seemed independent of VEGF uptake and VEGFR-2 is not responsible for VEGF uptake. We also demonstrate in vivo uptake of VEGF in *P. berghei* ANKA, analogous to the in vitro uptake in *P. falciparum*, making it possible to study the effects of VEGF signalling in vivo during a malaria infection.

## **The population dynamics of *Ascaridia galli* in chicken following trickle-treatment-challenge infection.**

Tania Ferdusky<sup>1</sup>, Torben W Schou<sup>3</sup>, Peter Nejsum<sup>1</sup>, Stig M Thamsborg<sup>1</sup>, Allan Roepstorff<sup>1</sup>, Liselotte R Norup<sup>2</sup>, Tina S Dalgaard<sup>2</sup>, Anders Permin<sup>3</sup>, Helle R Juul-Madsen<sup>2</sup>, Niels C Kyvsgaard<sup>1</sup>

<sup>1</sup>Faculty of Health and Medical Sciences, University of Copenhagen, Denmark; <sup>2</sup>Department of Science and Technology, Aarhus University, Denmark; <sup>3</sup>DHI, Environment and Toxicology, Denmark.

Control strategies of *Ascaridia galli* in chickens among many other factors may also depend on the level of acquired resistance against the parasite, and we therefore conducted the present trickle – treatment – challenge infection study. Sixty nine white Leghorn chickens of 18 weeks of age were randomly allocated into six experimental groups. Chickens belonging to group 1 were again divided into 3 sub-groups e.g. 1A (infected orally with 100 embryonated *A. galli* eggs and slaughtered at 3 days post infection, 1B and 1C (infected with 100 *A. galli* eggs twice weekly for 3 weeks and 6 weeks, respectively and slaughter 3 days after the last inoculation). All the birds belong to group 2, 3 and 4 were trickle infected with 100 eggs twice weekly for 6 weeks. Six weeks after the first infection chickens in Group 3 and 4 were treated with flubendazole for 7 days in the feed. Necropsy of the chickens in Group 3 confirmed that the treatment was 100% efficacious. Two weeks after treatment the chickens in group 4 were challenge infected with 500 *A. galli* eggs as were the chickens of a previously uninfected group (group 5). All the chickens were killed 1.5 weeks after challenge. At necropsy it was seen that the establishment of larvae increased initially and thereafter were decreasing with time despite getting higher number of eggs (group 1 and 2) and the chickens in the immunized group (group 4) had a lower number of *A. galli* compared to challenge-only chickens in group 5 (20 vs. 45,  $P < 0.05$ ). Also the larvae were smaller (mean sub-length 0.92 mm vs 1.63mm), they were located more posterior in the intestine and more larvae were located on the mucosa rather than in the intestinal content in the immunized group.

The study provides evidence that acquired immunity against *A. galli* in chickens leads to a significant yet incomplete protection against re-infection resulting in a decrease in establishment rate and an impaired development of the larvae.

## Eosinophil granule proteins ECP and EPX as diagnostic markers for active lesions in FGS

Anna Kildemoes<sup>1</sup>, Bodo Randrianasolo<sup>2</sup>, Charles-Emile Ramarokoto<sup>2</sup>, Pascaline Ravoniarimbina<sup>2</sup> and Birgitte J Vennervald<sup>1</sup>

<sup>1</sup>University of Copenhagen, <sup>2</sup>Pasteur Institute Madagascar

In recent years female genital schistosomiasis (FGS) has begun to receive attention, since genital lesions might exacerbate the risk of contracting sexually transmitted infections of which especially HIV represent a dire consequence. Genital epithelial granulomas induced by *Schistosoma haematobium* ova can manifest as three types of lesions visible by colposcopy; tubercle papules (TP), homologous yellow sandy patches (HYSP) and grainy sandy patches (GSP). It is well established that active *schistosome* egg granulomas are eosinophil rich and hence eosinophilia is a candidate diagnostic marker for active FGS lesions. Here it is investigated whether eosinophil specific proteins ECP and EPX in urine and lavage can be used as diagnostic markers for identifying active FGS lesions. Samples from 119 Madagascan women between 15 and 35 years of age are analysed by standard avidin/biotin amplified ELISA. 42 of the women carry the diagnosis FGS by colposcopy. Only samples from women with at least one TP lesion are shown to have significantly higher levels of ECP and EPX. Furthermore it is found that women with TP lesion are significantly younger, which indicates that TP lesion might be an “earlier” or “more active” inflammatory lesion stage. Results also support previous research pointing at ECP being a clearer indicator of lesions, as EPX levels seem to be innately higher in both urine and lavage representing a risk of “spill-over” between compartments. Measuring ECP in lavage might be a future tool for mapping FGS lesion onset and progression in order to determine, how early and frequently treatment must be administered in order to counter development of chronic inflammatory FGS lesions. Such a non-invasive tool is crucial in order to diagnose and investigate FGS as it seems measures must be taken in young females, where gynaecological examinations are not feasible or acceptable.

## **Difference in sensitivity of *Trichuris suis* and *Oesophagostomum dentatum* towards fenbendazole and albendazole in an in vitro assay**

Tina VA Hansen<sup>1</sup>, Annette Olsen<sup>1</sup>, Peter Nejsum<sup>1</sup>, Christian Friis<sup>2</sup>, Stig M Thamsborg<sup>1</sup>

<sup>1</sup>Department of Veterinary Disease Biology, Section of Parasitology, Health and Development;

<sup>2</sup>Department of Veterinary Disease Biology Section of Biomedicine, Faculty of Health and Medical Sciences, University of Copenhagen.

Background: The current control strategy against the whipworm of man (*Trichuris trichiura*) is administration of a single-dose of albendazole (ALB, 400 mg) or mebendazole (MBD, 500 mg), both having a low efficacy against *T. trichiura*.

*Trichuris suis* and *Oesophagostomum dentatum* are sharing the same habitat in the lower intestine of the pig, except that *O. dentatum* is a lumen dwelling nematode whereas *T. suis* is buried in the mucosa. In contrast to *T. suis*, *O. dentatum* is highly sensitive to benzimidazoles (BZs).

Objective: Using *T. suis* as a model for *T. trichiura*, the aim was to evaluate whether the difference in sensitivity towards BZs is due to a difference in the accumulation of BZs within the nematodes.

Method: *T. suis* and *O. dentatum* were isolated from experimentally infected pigs. The nematodes were incubated at 39 °C in a FBZ- or ALB concentration gradient (ranging from 0.01 to 30 µM) in RPMI medium. Control nematodes were incubated in RPMI + 2 % dimethyl sulfoxide (DMSO). Alive and dead parasites were incubated in 10 µM FBZ or ALB for 24 hours. Motility measurement was performed visually after 24, 48 and 72 using a motility scale ranging from 0 to 3. Nematodes were washed thoroughly, frozen in liquid nitrogen and kept at - 80 °C until high-performance liquid chromatography (HPLC).

Results: A clear tendency of *T. suis* being less sensitive than *O. dentatum* when exposed to FBZ ( $p = 0.052$ ) and ALB was observed. The accumulation of FBZ doubled for every 24 hour in *O. dentatum*, whereas in *T. suis* the concentration of FBZ was similar for parasites exposed to 1; 3; 10 and 30 µM. The accumulation of ALB was higher in *O. dentatum* than in after *T. suis* after 72 hours.

Conclusion: We found that the motility of *T. suis* was less affected by FBZ and ALB than *O. dentatum* and that *T. suis* accumulates less FBZ and ALB than *O. dentatum* over time. We therefore speculate that the low efficacy of BZs against *T. trichiura* might be due to low drug-up-take of the parasite.

## POSTERS

### **Expression and purification of PFD1235w-DBLb3 hybrids in *E.coli* and characterization of ICAM1 binding properties**

Rebekka W Olsen, Andersen MA, Barbati ZR, Bengtsson A, Jørgensen L and Jensen ATR  
Center for Medical Parasitology, Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen and Department of Infectious Diseases, Copenhagen University Hospital (Rigshospitalet)

*Plasmodium falciparum* is the most pathogenic human malaria parasite and its virulence has been linked to its capacity to express different adhesion proteins that enable the infected erythrocyte to bind to capillaries of the human host and thereby avoiding removal by the spleen. The best characterized family of adhesion proteins is *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) of which some variants such as PFD1235w have been associated with severe malaria. PFD1235w encompass several different subdomains and of particular interest are two domains of the subtype DBLb3 of which only one can bind to the host receptor ICAM1. To determine which region of the ICAM1 binding DBLb3 is responsible for the ICAM1 binding we have in an earlier study divided each of the two different DBLb3 regions into three and shuffled them to produce 6 different hybrid molecules using the baculovirus/insectcell expression system. Since the yield of purified hybrid molecules was often poor using this system we have moved our constructs to an *E. coli* expression system. In this study the hybrids were expressed in *E. coli* and purified by metal-ion affinity chromatography followed by anion and cation exchange chromatography. The purified hybrids will be characterized with respect to ICAM1 binding abilities and be compared to the hybrids expressed in the baculovirus/insectcell system."

## Prevalence of *Taenia hydatigena* cysticercosis in Mbeya Region, Tanzania

Michelle Christensen<sup>1</sup>, Peter Nejsun<sup>1</sup>, Helena Ngowi<sup>2</sup>, and Maria V Johansen<sup>1</sup>

<sup>1</sup>University of Copenhagen; <sup>2</sup>Sokoine University of Agriculture, Tanzania

In small ruminants, *Taenia hydatigena* may result in revenue loss for smallholders through disease and condemnation of livers. The aim of the study was to determine the prevalence of *T. hydatigena* in small ruminants in Mbeya region, Tanzania, and to establish the identity in a subsample of metacestodes by DNA sequencing of the cytochrome c oxidase subunit 1 gene (cox1). In autumn 2012, goats and sheep slaughtered at Mbalizi slaughter slab in Mbeya Region were inspected for the presence of *T. hydatigena* metacestodes. A random subsample of cysticerci suspected to be *T. hydatigena* were collected and stored in 70 % ethanol. DNA was extracted (n=12) and the cox1 gene was PCR amplified and sequenced followed by BLAST search (GenBank) and cluster analysis using MEGA5.

In 392 goats and 27 sheep, the prevalence of *T. hydatigena* was 45.7 % (CI 40.7-50.6%) and 51.9 % (CI 33.1-70.7%), respectively. All PCR products were sequenced and identified as *T. hydatigena* by BLAST search in GenBank. This finding was supported by cluster analysis. Sequence information on *T. hydatigena*, *T. saginata*, *T. taeniaeformis*, *T. solium* and *T. multiceps* were used to construct a Neighbour-Joining tree and all *T. hydatigena* sequences were in one cluster with very little distance between isolates (p-distance: 0.000-0.028) and distinct from other taeniids included in the tree (p-distance: 0.055 – 0.163).

In conclusion, *T. hydatigena* is highly prevalent in the goats and sheep in Mbeya region, and could be responsible for significant smallholder revenue loss; however this needs further study to determine. Pigs could harbour *T. hydatigena* in Mbeya region, and this should be investigated, since *T. hydatigena* in pigs may be a source of false-positives in genus-specific serological tests for *T. solium*.

Based on cox1 sequence analysis we found that all 12 metacestodes were *T. hydatigena* and suggest that this might be a useful genetic marker for differentiation between *T. hydatigena* and other taeniids.



### **A genetic marker allele conferring resistance to *Ascaris suum* in pigs**

Per Skallerup<sup>1,2</sup>, Stig M Thamsborg<sup>1</sup>, Claus B Jørgensen<sup>2</sup>, Heidi L Enemark<sup>3</sup>, Ayako Yoshida<sup>4</sup>, Harald HH Göring<sup>5</sup>, Merete Fredholm<sup>2</sup> and Peter Nejsum<sup>1,2</sup>

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Mapping of quantitative trait loci (QTLs) has helped dissecting the genetics underlying the variation in resistance to helminth infections. In pigs, two single nucleotide polymorphisms (SNP TXNIP and SNP ARNT), both on chromosome 4, have been reported to be associated with *Ascaris suum* worm burden. In the present study we selected pigs with two SNP TXNIP genotypes (AA; N=24 and AB; N=24) which from eight weeks of age were experimentally infected with *A. suum* until necropsy at week 8 post first infection (p.i.) to test the hypothesis that pigs with the AA genotype would have lower worm burdens than pigs of the AB genotype. We used different indicators of resistance (worm burden, faecal egg counts, number of liver white spots and *A. suum*-specific serum IgG antibody levels) of which the first two traits were considered core traits and the last two traits were associated traits. Pigs of the AA genotype had lower mean macroscopic worm burden (2.4 vs. 19.3), lower mean total worm burden (26.5 vs. 70.1) and excreted fewer *A. suum* eggs at week 8 p.i. (mean number of eggs/g faeces: 238 vs. 1259) than pigs of the AB genotype. However, none of these differences were significant (P-values of 0.06, 0.06 and 0.14, respectively). In addition, pigs of the AA genotype had lower (though not significant) serum IgG antibody titres to three different *A. suum* antigens. There was no significant difference between genotypes in the number of liver white spots. The pigs were also genotyped at another locus (SNP ARNT) which showed a similar trend. The data presented here provide suggestive evidence that resistant pigs can be selected using a genetic marker, TXNIP, and that it is the B allele which is conferring susceptibility to *A. suum* infection. Our work confirmed that SNP ARNT is another diagnostic marker candidate for *A. suum* resistance and provides further support to the QTL on porcine chromosome 4 detected previously.

## **Prevalence of the protozoan parasite *Cryptosporidium* on three organic pig farms in Denmark**

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Pigs are a potential source of contamination with *Cryptosporidium* spp., which can lead to infection in humans. Two species *C. parvum* and *C. hominis* can cause an acute diarrheal illness in humans, which can become severe in e.g. patients with HIV. The oocyst can survive for long periods in the environment and is resistant to many common disinfectants. In order to estimate the prevalence of the zoonotic parasite *Cryptosporidium* in organic pigs and to improve our knowledge of the parasite epidemiology, the prevalence was monitored four times between September 2011 and June 2012 in three Danish organic pig farms. Faecal samples were collected for examination of *Cryptosporidium* spp. with a total of 994 pigs grouped as sows, fatteners, young pigs and piglets. The number of pigs in each age group was 298, 232, 315 and 161 respectively, distributed on the three farms. Faecal samples were collected four times and oocysts were counted by microscopy. The overall prevalence of *Cryptosporidium* spp. was 38%. The prevalence in the four age groups was very different, with 3% of the sows, 40% of the fatteners, 65% of the young pigs and 45% of the piglets found positive. There was no noteworthy difference in the overall prevalence between farms (33%-42%) or between the four sampling times (33%-42%). The intensity of the *Cryptosporidium* infection was dependent on the age of the pigs, with the younger having the highest OPG. Only in the young pigs and the piglets more than 105 OPG was seen and the 10 pigs with the highest OPG were all piglets. There were no noteworthy differences in the intensity of the *Cryptosporidium* infection between the four sampling times, but there was an apparent association between the number of positive sows and the number of piglets with high OPG, since 9/10 pigs with the highest OPG were found on the same farm which also had the highest prevalence in sows. Further studies on the species and zoonotic potential of the *Cryptosporidium* oocysts are ongoing.

## Developing a laboratory method to assess the number of *Ascaris* eggs on hands

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*Ascariasis* are transmitted through the faecal-oral route; eggs are ingested following contact with contaminated food, soil or the deliberate act of eating contaminated soil. *Ascaris lumbricoides* has a low infective dose: a single egg can cause an infection. Infective *A. lumbricoides* eggs can survive and remain infective for several months in the environment. Eggs have been found on vegetables, particularly in areas where human faeces or wastewater are used as fertilizer, and eggs may be directly ingested during meals if produce are not washed. *A. lumbricoides* eggs are most commonly found in soil, but also on banknotes, from where they may transfer to hands and be ingested. The role of hands in the transmission of *A. lumbricoides* eggs is unclear, as studies on the subject are lacking. The present study aimed at developing a laboratory method that would allow counting of *A. lumbricoides* and possibly other helminth eggs present on hands, and assessing its recovery rate. Hands of 6 volunteers were contaminated with 1000 *Ascaris suum* eggs, which are used as a model for *A. lumbricoides* eggs since the eggs are morphological identical. Three different rinsing solutions was tested, i.e. de-ionized water, 1% 7X (non-ionic), and 0.1% Benzethonium Chloride (cationic). Contaminated hands were washed inside bags containing the rinsing solution and the number of eggs in the rinsing water was enumerated using McMaster slides. The average recovery rate is estimated to 95.6% (95% CI 89.6 - 100) for 7X, 89.3% (95% CI 81.0 - 97.7) for de-ionized water and 88.2% (95% CI 79.2 - 97.2) for Benzethonium Chloride. There is no statistically significant difference across these numbers. This indicates that just by washing hands with water will reduce the number of *Ascaris* eggs on hands, and thereby reduce the risk of ingestion.

### **Migratory pattern of *Toxocara cati* and *Toxocara canis* in pigs**

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In man infections with *Toxocara cati* and *T. canis* may cause visceral larva migrans (VLM). The relative contribution of the two species in VLM as well as the predilection sites of *T. cati* in the human host is largely unknown. We used the pig as a model for human infection and conducted two studies to explore the migratory patterns of *T. cati* as compared to *T. canis*. Four groups of 5-7 pigs were inoculated with 50.000 (study A) or 10.000 (study B) embryonated eggs of either *T. cati* or *T. canis* eggs. Larvae were recovered from liver, lungs, mesenteric lymph nodes, heart, brain, eyes, diaphragm, tongue and skeletal muscles at day 14 post infection (study A) or day 30-32 p.i. (study B) by HCl/pepsin digestion. A higher, but not significantly so, mean larval burden was found in lymph nodes of *T. cati* than *T. canis*, both on day 14 p.i. (122.0 vs. 90.0) and day 30-32 p.i. (3.6 vs. 1.3). The opposite trend was found in the lungs, both on day 14 p.i. (13.8 vs. 22.0; NS) and on day 30-32 p.i. (0.6 vs. 3.0; P=0.07). In addition, we found larvae in three of the *T. canis* infected pigs (1, 6 and 9 larvae) on day 14 p.i. and 1 out of 7 on day 30-32 p.i. (1 larva) whereas no larvae were found in livers of *T. cati* infected pigs. On day 30-32 p.i., two larvae were recovered from skeletal muscles of a *T. cati* infected pig and one larva from the diaphragm of a *T. canis* infected pig. Haematology showed more pronounced eosinophilia in *T. canis* compared to *T. cati* infected pigs on day 22 p.i. in study B. Our data suggest a different tissue distribution of *T. cati* and *T. canis* which may reflect different migratory patterns in VLM and perhaps different pathogenicity.

**Efficacy and Safety of Dihydroartemisinin-piperaquine for treatment of uncomplicated falciparum malaria in pregnancy in Ghana ; a randomised, open-label, non-inferiority trial.**

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Pregnancy-associated malaria is a challenge in endemic areas with adverse outcomes for both mother and foetus. One intervention is effective case management with artemisinin-based combination therapy. One such option is dihydroartemisinin-piperaquine (DHA-PPQ) for which there is limited efficacy and safety data in pregnancy. With its introduction in Ghana and anticipated access by pregnant women, we assessed its use for treatment in pregnancy. Pregnant women of gestation 15-32 weeks attending antenatal clinics in a moderate-to-high transmission zone were screened for *falciparum* parasitaemia using rapid diagnostic test (RDT) and microscopy. Those positive in both and eligible were randomized to receive dihydroartemisinin-piperaquine or artesunate-amodiaquine (AS-AQ). Baseline clinical and haematological assessments are conducted. They were actively followed up on days 1, 2, 3, 7, 14, 28, 42, at delivery and at 6 weeks postpartum to ensure adherence to study drugs, assess adverse events, collect blood samples for haematological and parasitological assessments and gather data on neonatal morbidity and mortality. Parasite clearance at day 28 was 99.4% in the DHA-PPQ group and 98.0% in the AS-AQ group with a risk difference of 1.4% and a two-sided 95% CI -1.13-3.93 falling within the 5% margin of non-inferiority. There was no difference in mean haemoglobin concentration between groups at day 28 (10.5 vrs 10.7; p=0.113) but a significant reduction in the DHA-PPQ group at day 42 (10.1 vrs 10.6; p<0.001). Differential counts were similar on days 28 and 42. Higher prevalences of adverse events were noted on day 3 in the AS-AQ group; anorexia (12.4% vrs 21.0%; p=0.03), dizziness (12.9% vrs 26.7%; p=0.003) and general weakness (39.3% vrs 61.5%; p<0.001). We will compare PCR-corrected parasitological efficacy at days 28 and 42, low birth weight, maternal anaemia, adverse events and foetal loss in the two treatment arms for significance of differences.

## ***Dientamoeba fragilis* in Denmark: Epidemiological experience derived from four years of routine real-time PCR**

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The clinical significance of the common intestinal flagellate *Dientamoeba fragilis* remains uncertain, and while several studies have addressed aspects of epidemiology, only a handful have reported on observations generated by the use of standardisable and highly sensitive real-time PCR. The aim of this study was to report on results and experience gained by testing more than 22,000 samples using from the period 2008—2011. We demonstrate that an average of 42.7 % of tested patients were positive for *D. fragilis*, ranging from 10 to 70% depending on age group, with a bimodal age distribution with peaks in children and adults of parental age, particularly women, indicating association between exposure to children and risk of *D. fragilis* infection. These findings further substantiate our knowledge of risk factors pertaining to *D. fragilis* carriage, and are discussed in light of the pinworm vector transmission hypothesis.

**Reappearance of *Taenia ovis krabbei* muscle cysts in a roe deer (*Capreolus capreolus*) in Denmark after 60+ years, with a possible role of a grey wolf (*Canis lupus*) as definitive host**

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*Taenia ovis krabbei* is a parasite with a sylvatic life cycle in which carnivores are definitive hosts and Cervid are intermediate hosts. Foraging on pasture contaminated with eggs of *T. o. krabbei* is the primary cause of infection to Cervids, and the larval stages usually develop in heart and skeletal muscles causing pathological changes and severe illness. There is no zoonotic risk in consumption of game meat infected with *T. o. krabbei*, but for aesthetic reasons, the infected meat is not regarded of high quality and usually discarded. The present report describes the reappearance of *T. o. krabbei* in a roe deer Denmark after more than 60 years. The cysticerci were isolated from the thigh muscle of a male roe deer shot in northern Jutland, and the diagnosis was based on histological analysis, morphology of the rostellar-hooks as well as molecular typing of the mitochondrial cytochrome c oxidase I (cox1) gene. Shortly after this discovery, a wolf died in a nearby locality and worms *T. o. krabbei* was recovered from its intestine, and the diagnosis was based on morphology of the rostellar-hooks and molecular typing of the cox1 gene. By phylogenetic analysis, the Danish roe deer and wolf isolates were clearly grouped together with other isolates of *T. o. krabbei* from wolves in Finno-scandinavia. In mainland Europe, *T. o. krabbei* is primarily a parasite of wolves and this individual wolf has probably travelled around 800 km before it died. This unexpected reappearance of a wolf in Denmark after almost two decades could be a mere coincidence, but can also explain the introduction of this parasite during wolf introduction. Domestic dogs, in the other hand, could play a role in transmission of *T. o. krabbei* in that area, but this has yet to be tested. Deer infections with *T. o. krabbei* were reported in all German counties that border Denmark. It is also possible that similar deer infections were already present in other areas in Denmark, but unnoticed.

## **Anthelmintic-resistant strains of *Haemonchus contortus* and *Trichostrongylus colubriformis* isolated from an organic sheep and goat farm in Denmark**

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A suspected case of anthelmintic resistance (AR) was investigated in an organic dairy sheep and goat farm. The herd was established in 2007 by purchase of animals from a number of other farms. Selection for the study was based on history of anthelmintic-treatment failure. Forty-eight lambs and 48 kids were selected for faecal egg count (FEC) reduction tests. Animals were allocated into one of 5 treatment groups, or 1 untreated control group, for each species. Lambs were treated with 5 mg/kg fenbendazole (FBZ), 0.2 mg/kg moxidectin (MOX), 7 mg/kg levamisole (LEV), 0.2 mg/kg ivermectin (IVM) or 10 mg/kg closantel (CLO). Kids were treated with 10 mg/kg FBZ, 0.3 mg/kg MOX, 14 mg/kg LEV, 0.2 mg/kg IVM or 10 mg/kg CLO. FECs were performed at day of treatment and 14 days post treatment. In a subsequent investigation, faeces from adult goats were cultured to obtain 3rd-stage nematode larvae (L3) for a controlled efficacy test (CET). The resulting isolate was composed of 77 % *Trichostrongylus/Teladorsagia* spp. and 33 % *Haemonchus* spp. Eighteen parasite-naïve lambs were infected with 9,000 L3/lamb of this isolate. At day 35 post-infection (p.i) lambs were allocated in 3 groups and treated with 5 mg/kg FBZ, 0.2 mg/kg IVM or left untreated as controls. At day 42 p.i. lambs were euthanized and worms recovered for species identification. In the field study, FBZ reduced the FEC of lambs and kids only by 27 % and 56 %, respectively, and IVM reduced the FEC in lambs and kids by 71% and 81 %, correspondingly. In the CET, FBZ reduced *H. contortus* worm burdens by 52 %, whereas *T. colubriformis* was not affected by the treatment, and worm counts were not significantly different from the untreated control group ( $p > 0.05$ ). IVM removed 100 % of adult *H. contortus* and reduced *T. colubriformis* worm counts by 84 %. This is the first isolation of BZ-resistant *H. contortus* and *T. colubriformis* in Denmark and highlights the need for continuous surveillance of AR in conventional and organic farms.



## Evaluation of soil microfungi as biological control agents against ascarid eggs

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Thick-shelled ascarid eggs have been reported to remain infective in the environment for several years, thus posing a prolonged risk of infection to the animal or human hosts. The following *in vitro* study was therefore conducted to evaluate the negative impact of two species of soil microfungi (*Pochonia chlamydosporia* (Pc) and *Paecilomyces lilacinus* (Pl)), on the viability of *Ascaridia galli*, *Toxocara canis* and *Ascaris suum* eggs. For each parasite, three experimental groups (control, Pc and Pl) each consisting of eighteen Petri dishes were maintained. Approximately 150 fresh eggs of individual ascarid species were embryonated on a 2% water agar in Petri dishes with or without a fungus (*P. chlamydosporia* or *P. lilacinus*). On days 7, 14, 21, 28, 35 and 42 post experimental set up (p.s.), the resulting viability of the eggs in three Petri dishes from each experimental group was evaluated (destructive sampling). By day 14 p.s., *P. chlamydosporia* had reduced the viability of *A. galli* and *T. canis* eggs by 70-86% and 52-67%, respectively, compared to the controls. In contrast, *P. lilacinus* had reduced the viability of *A. galli* and *T. canis* eggs by only 17-30% and 6-28%, respectively. Neither fungal species was found to be effective against *A. suum* eggs (<4% reduction in both cases). These results indicate interspecies differences in the susceptibility of ascarid eggs to microfungi. *Ascaridia galli* and *T. canis* eggs seemed to have been degenerated mainly due to hydrolysis of shells by fungal enzymes. In contrast, mechanical penetration of shells by fungal hyphae appeared to be the primary mode of *A. suum* egg degeneration. The present study demonstrates that *P. chlamydosporia* may potentially be utilized as a biological control agent in reducing *A. galli* and *T. canis* egg contaminations in the environment.