JOINT SPRING SYMPOSIUM 2012

"Double burden of disease – how parasites interact with each other, their host and the society"

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

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Friday 23rd of March, 2012, 8:00-16:10

Faculty of Life Sciences, Lecture room 1-01 (Festauditoriet)
Bülowsvej 17, 1870 Frederiksberg C

ORAL PRESENTATIONS – KEY NOTES

Using a One Health approach addressing the multiple burden of Taenia solium cysticercosis in sub-Saharan Africa

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As the human population continues to increase and expand across the world, the interconnection of people, animals, and the environment becomes more significant and impactful. A significant outcome has been the increase in emerging diseases in recent time. Among the 177 identified emerging and re-emerging human diseases, 73% are zoonoses. However, the impact and control of these diseases are grossly neglected as the magnitude of the dual burden, which these diseases impose on human and animal health, remain to be elucidated. As a global response to the increase in emerging diseases and in particular to the avian influenza epidemic, international organizations recognized the need for strengthening intersectoral collaboration. This led to the revival of the 'One Health' concept. The definition of 'One Health' is still up for debate but the definition: '*the collaborative efforts of multiple disciplines working locally, nationally and globally to attain optimal health for people, animals and our environment'* is getting more and more widely accepted.

In 2010 WHO published the first report on neglected tropical diseases. Seventeen priority diseases were listed of which 6 are zoonoses including three with helminth aetiology, i.e. *Taenia solium* cysticercosis, foodborne trematodiasis and echinococcosis. The strategy for control of these diseases includes burden assessments, using a One Health approach and applying multi-intervention disease control packages.

T. solium cysticercosis was declared eradicable in 1993, but in eastern and southern Africa (EAS) the disease has been emerging in the past decade and little attention has been paid to its control. At the same time pig keeping and pork consumption have increased significantly in the region especially in rural smallholder communities. A high and increasing prevalence of epilepsy in ESA, without a clear etiology, and the appearance and increase in cases of porcine cysticercosis have been noted in the region. A Danida-funded research project was implemented from 2006 – 2009 in Mozambique and Tanzania with the aim to assess the prevalence, risks and impacts of *T. solium* taeniosis/cysticercosis in both humans and pigs. The studies were conducted in Mbeya region, southern highlands of Tanzania and in Angonia district, Tete province, northern Mozambique. Quantitative methods were applied i.e. Ag-ELISA for both human and porcine cysticercosis, Ab-ELISA for human cysticercosis, and CT scans for presence of human brain cysts. Qualitative methods were used to assess perceptions, attitudes and practices of community members. In Tanzania, 31% and in Mozambique 35% of the pigs were *T. solium* positive. In Tanzania 17% of humans were Ag-positive whereas 45% were Ab-positive for cysticercosis. In Mozambique, 15% were Ag-positive. Among a subgroup of the study subjects in Mozambique, 72% Ag-positive compared to 18% Ag-negative persons were having abnormal CT scans suggestive of neurocysticercosis. Epilepsy was in both countries very common and strongly associated with stigmatisation. Risk factors for *T. solium* infections included poor pig husbandry practices especially free raging pigs, open defecation, age of pigs, pork cooking practices, lack of meat inspection, and lack of knowledge regarding the transmission of the disease. People were aware of 'white nodules' in pigs and knew of epilepsy, but had no idea of the linkage. The studies in Mozambique and Tanzania indicate that *T. solium* taeniosis/cysticercosis is endemic in the region and causes severe health and economical problems to the rural smallholder communities. An on-going Danida funded project is now trying to assess the true societal cost of this disease and suggesting evidence-based safe, humane and profitable smallholder pig production model systems.

Double burden of infectious and non-communicable diseases, emphasis on parasites and NCD

Bygbjerg I.

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Parasites had a renaissance when crypto-, iso-, micro-sporidioses, leishmaniasis, toxoplasmosis and even malaria met with HIV. What may happen when parasites and other infections meet with non-communicable diseases?

ORAL PRESENTATIONS

Effects of hydrogen sulfide donors on malaria parasite growth and development of experimental cerebral malaria.

Della Valle B. Staalsoe T. Kurtzhals JAL, Hempel C.

Hydrogen sulfide (HS) is a physiological gas involved in biological signalling with many overlapping functions with two other known physiological gases, carbon monoxide (CO) and nitric oxide (NO). Interestingly, experimentally boosting CO or NO levels protects mice from developing experimental cerebral malaria (ECM). Moreover, treatment with exogenous HS gas or HS donor molecules protects the brain from damage in rodent models of neurodegeneration and oxidative damage. Finally, previously published data suggests that HS may significantly inhibit parasite growth. This study was thus designed to investigate the therapeutic potential of HS treatment in a model of ECM and its effect on parasite growth both *in vitro* and *in vivo*.
In vitro: *Plasmiodium falciparum* was grown in culture media supplemented with saline or increasing doses of two different HS donors. Parasitemia was quantified after 48 hours.
In vivo: Over the course of three independent studies, HS treatment was initiated with either a fast-releasing donor or slow-releasing donor at day 4 post-inoculation using a wide spectrum of dosing regimes. Prognosis, parasite growth, and plasma thiols were quantified.
In vitro, both fast- and slow- releasing HS donors were effective in inhibiting *P. falciparum* growth in a dose-dependent manner. In all three separate *in vivo* studies, no combination of HS donor and dosing regime delayed the onset of symptoms, parasite growth and ultimately the fatal outcome of ECM. Interestingly, HS treatment did not significantly increase plasma thiol content and thus may provide an explanation for the absence of *in vivo* efficacy.
In conclusion, these results suggest that *in vivo* HS treatment in this manner is likely not an adjunctive therapy option for the treatment of cerebral malaria. Interestingly, the effect of HS on parasite growth *in vitro* and previous work on positive HS effects in the brain suggest further investigation with inhaled HS could be pursued to exploit potential therapeutic effects of HS.

Invertebrate-parasite models: determining infection reliability in relation to methodology and dose and subsequent suitability for pharmacological testing.

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A method of administration of parasite eggs to *Tenebro molitor* was developed testing two different ways of exposing the beetles to infective material and evaluated for the effect on cysticercoids establishing. Suspensions of *Hymenolepis diminuta* eggs were prepared at four different concentrations (10, 50, 100 and 150 eggs per 10 µl droplet). Beetles received either a single droplet of egg suspension or a droplet administered to on rolled oat. The results suggest that the single droplet administration incurs highest infection rates. There also seemed to be a dose-response in both administration methods. The method ensures reliable infection levels of intermediate hosts. Cysticercoids were also exposed to one of three drugs, Praziquantel, Mebendazole (two drugs with known efficacy against cestodes on the mammalian system) and Tetramisole (a negative control – limited efficacy with tapeworms) *in vitro* at five separate concentrations (0.05, 0.005, 0.0005 0.00005 and 0%) for a 1-hour period. After exposure, an excystation procedure was performed and the cysts were subsequently observed at 60-minute intervals for a period of 3 hours. In relation to the negative control, praziquantel and mebendazole achieved more excystation inhibition ($P<0.0001$ and 0.0004 respectively). There was also an effect of concentration; at 60 and 180 minutes the 0% concentration achieved significantly more excystation than 0.05% (<0.0001, <0.0001). These results demonstrate that *in vitro* insect models could hold promise for pharmaceutical testing. Prospective study will be focused on *in vivo* models and toxicological testing in the insect system.

The Comparison of Efficacy of Ivermectin and Oxfendazole for Treatment of Porcine Cysticercosis in Naturally Infected Pigs.

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An experimental treatment trial was conducted to evaluate the efficacy of ivermectin (IVM) in comparison to that of oxfendazole (OFZ) in treatment of muscular and brain porcine cysticercosis. A total of 60 naturally infected pigs as identified by tongue palpation of both sexes with age ranging from 3 – 24 months were recruited. The pigs were stratified based on sex, age and cysts intensity as judged by tongue examination and then randomly allocated in IVM, OFZ and control groups. Blood sample was taken before and every two weeks after treatment for detection of *T. solium* circulating antigen in response to treatments. The results of viability study showed that IVM had little effect on metacestodes but was not significant ($p > 0.05$) in comparison to the control group. Significant effect observed in the OFZ treated group ($p < 0.05$) in comparison to IVM and control group with exception to brain metacestodes which showed no significant difference ($p > 0.05$) between the three groups. The IVM at single dose of 0.03 mg/kg body weight is not effective in control of porcine cysticercosis as compared to single dose of 30 mg/kg OFZ which killed all metacestodes except those in the brain as early as four weeks after treatment, but more than twelve weeks required for clearance of metacestodes as calcified cysts were visible in ten out of twelve carcasses in this group twelve weeks after treatment.

A molecular basis for differing Ivermectin sensitivity in vertebrate, Platyhelminth and Nematode cys-loop receptors.

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Ivermectin is a potent nematicide, insecticide and acaricide because it inhibits signalling in nematode and arthropod nervous systems. It does so by potently activating inhibitory ligand-gated ion channels of the Cys-loop receptor family, namely the glutamate-gated chloride channel (GluCl). Ivermectin also activates vertebrate Cys-loop receptors but with much less potency, making ivermectin safe to mammals. The question remains as to why platyhelminths are not susceptible to ivermectin. The ivermectin binding site in these receptors has long been unknown. We used site-directed mutagenesis, electrophysiology and computer-aided molecular modelling to describe the ivermectin binding site in the vertebrate glycine receptor (GlyR). Our results showed that ivermectin binds to a membrane-embedded site of the GlyR, similar to the site in the nematode GluCl, revealing the amino acid determinants of ivermectin sensitivity. We then compared the amino acid sequences of characterised Cys-loop receptors from nematodes and vertebrates and to those from uncharacterised transcripts from recent platyhelminth genomes. Our comparison showed that platyhelminth genomes possess GluCl-like transcripts, but these differ from nematode GluCls in key positions that determine ivermectin sensitivity. Thus, our results suggest that platyhelminths possess GluCls, provide a means of predicting ivermectin sensitivity and provide a structural basis for developing novel antiparasitic drugs that target platyhelminths.

Determining prevalence of cyp2b6 alleles in Tanzania using a using a post-PCR ligation detection reaction-fluorescent microsphere assay.

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Objective: Cytochrome P450 2B6 (CYP2B6) variants have been shown to affect metabolism of a variety of drugs including the artemisinin drugs used to treat *falciparum* malaria, and as well some anti-retrovirals and anti-tuberculosis drugs. The aim of this study was to determine the prevalence of the most commonly observed CYP2B6 alleles in a malaria- and HIV-endemic population in Tanzania.
Methods: DNA samples were collected from Tanzanian individuals (n=51) enrolled in the study: "Interactions between ACTs for malaria and ARVs for HIV/AIDS" at Muheza Designated District Hospital in Muheza district, Tanga, Tanzania. Frequencies of the CYP2B6 alleles; *1,*2,*3,*4,*5,*6,*7 and *9 were determined using a post-PCR ligation detection reaction-fluorescent microsphere assay.
Results: Findings were compared with an earlier study from West Africa. Significant differences were found between the two populations for the frequencies of alleles *1 (20% vs. 45%, P=0.0003), *5 (16% vs. 2%, P=0.0008), *6 (27% vs. 42%, P=0.04). *7 were found in Tanzania but not in West Africa and *9 was not found in Tanzania. Alleles *3 and *4 were not found in either studies.
Conclusion: Several of the alleles known to affect the metabolism of different drugs were found in this study. Significant genetic differences were found compared to West Africa. It is important to determine if the genetic differences are associated with altered metabolism of artemisinins and other widely-used anti-infective drugs.

Confirmation of a QTL region associated with susceptibility to *Ascaris suum* infections in pigs.

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Helminths almost invariably have an overdispersed distribution in the host population. Human and animal studies have provided evidence suggesting that a large part of this variation is due to host genetic factors. We used single nucleotide polymorphism (SNP) markers to perform a whole-genome scan on 195 pigs experimentally infected with *A. suum*. A putative quantitative trait locus (QTL) for worm burden on SSC4 covering ~2.5 Mbp was identified by measured genotype analysis, although none of the SNPs reached genome-wide significance. To validate the putative QTL region, we genotyped two of the SNPs within the region in unrelated, informative animals exposed to experimental or natural infections and from which we had worm counts and/or faecal egg counts; the validation studies showed that one of the SNPs (TXNIP) was associated with total worm burden ($P<0.001$) and adult worm burden ($P<0.0001$), whereas the other SNP (ARNT) was associated with adult worm burden ($P<0.025$) in these populations. We were thus able to confirm the existence of the QTL on SSC4. This is to our knowledge the first report of a QTL associated with helminth burden in pigs.

DNA of *Dientamoeba fragilis* detected within surface-sterilized eggs of *Enterobius vermicularis.*

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Objectives: Numerous studies have suggested that eggs of intestinal nematodes, primarily *Enterobius vermicularis* (pinworms), can serve as vectors for *Dientamoeba fragilis*, an intestinal protozoan of suggested pathogenicity and common occurrence. Attempts to culture *D. fragilis* from pinworm eggs have however been unsuccessful, and contrasting findings have been found in studies on *D. fragilis*/pinworm co-infection. The aim of this study was to investigate whether we could amplify *D. fragilis* -specific DNA sequences from surface-sterilized pinworm eggs. Methods: Pinworm eggs were collected from routine diagnostic samples (cellophane tape) and surface-sterilizes these in a 1% HOCl (hypochlorite) solution. DNA was extracted from individual eggs, and conventional PCR performed using *D. fragilis* and *E. vermicularis* specific primers. Amplified products were sequenced for confirmation. Results: In cellophane tape samples from patients with unknown *D. fragilis* status we detected *D. fragilis* DNA in 12/238 (5 %) of the eggs, and in a patient known to harbour *D. fragilis* we detected *D. fragilis* DNA in 14/99 (14 %) of the eggs. Conclusion: The finding of DF-specific DNA sequences within EV eggs strongly support the hypothesis of DF-transmission by EV-vector, and have implications for antimicrobial interventions, as well as control and public health measures.

Genetic variation in mitochondrial DNA among *Enterobius vermicularis* in Denmark

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Objectives: Despite being one of the most prevalent nematode infections of man in the Western Europe and North America, our knowledge of the genetic variability in *Enterobius vermicularis* is fragmented. We here report on a genetic study of pinworms in Denmark, performed using the cytochrome oxidase I (cox1) gene, with DNA extracted from individual eggs collected from clinical (human) samples.
Methods: We collected cellophane-tape-test samples positive for pinworm eggs from 14 Departments of Clinical Microbiology in Denmark, and surface-sterilized the eggs using a 1% hypochlorite solution before performing conventional PCR and sequencing.
Results: 22 haplotypes were identified from a total of 58 Danish patients. Cluster analysis showed that all Danish worms grouped together with human samples from Germany and Greece and with samples from Japanese chimpanzees designated 'Type B'. Analysis of molecular variance showed no significant difference or trends in geographical distribution of the haplotypes in Denmark, and several haplotypes were identical or closely related to samples collected in Germany, Greece and Japan. However, worms from the 4 countries were found to belong to different populations, with Fst values in the range of 0.16 to 0.47.
Conclusion: This is the first study on genetic variation of pinworms in Denmark, using DNA isolated from single surface-sterilized pinworm eggs, showing a homogenous genetic population. Despite belonging to different populations, humans in Denmark, Germany and Greece were found to be infected with the same main worm haplotype ('Type B').

Classification of parasite eggs used as an active pharmaceutical ingredient (API).

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The aim of the project is to describe the developmental stages of parasite eggs using digital image analysis of multispectral imagery. The primary focus is to identify and count eggs of different developmental stages and distinguish them from unrelated particles. This information is used to assess the pharmaceutical potency of eggs from the parasite *Trichuris suis* (pig whipworm), used as an active pharmaceutical ingredient in medicine against chronic autoimmune diseases of the intestines such as Crohn's disease and Ulcerative colitis.
The analysis will combine spectral and morphological characteristics in order to first detect and then classify the parasite eggs based on a set of biologically inspired, quantitative features. The egg classification is currently done manually using transmitted light microscopy, but to allow for validation of this process and to reduce the operator bias, an automated system is being developed. The developed procedure should be robust, non-invasive and fast, and could potentially have applications in description of other organisms.
An initial analysis was carried out as a proof of concept. The detection of embryonated eggs was done using matched filtering and the classification was based on the orientation of the larva inside the egg: The ratio of longitudinal vs. latitudinal 'lines' inside the egg is higher for eggs containing a larva.

Use of *Moringa oleifera* seed extracts to reduce helminth egg numbers and turbidity in irrigation water.

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Water from wastewater-polluted streams and dug-outs is the most commonly used water source for irrigation in urban farming in Ghana, but helminth parasite eggs in the water represent health risks when used for crop production. Conventional water treatment is expensive, requires advanced technology and often breaks down in less developed countries so low cost interventions are needed. Field and laboratory based trials were carried out in order to investigate the effect of the natural coagulant *Moringa oleifera* (MO) seed extracts in reducing helminh eggs and turbidity in irrigation water, turbid water, wastewater and tap water. In medium to high turbid water MO extracts were effective in reducing the number of helminth eggs by 94-99.5% to 1-2 eggs per litre and the turbidity to 7-11 NTU which is an 85-96% reduction. MO is readily available in many tropical countries and can be used by farmers to treat high turbid water for irrigation, however, additional improvements of water quality, e.g. by sand filtration, is suggested to meet the guideline value of ≤ 1 helminth egg per litre and a turbidity of ≤ 5 NTU as recommended by World Health Organization. A positive correlation was established between reduction in turbidity and helminth eggs in irrigation water, turbid water and wastewater treated with MO. This indicates that helminth eggs attach to suspended particles and/or flocs facilitated by MO in the water, and that turbidity and helminth eggs are reduced with the settling flocs. However, more experiments with water samples containing naturally occurring helminth eggs are needed to establish whether turbidity can be used as a proxy for helminth eggs.

Parasites of the raccoon dog – an invading species.

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Invasive species have a marked negative influence on the biodiversity of ecosystems and may contribute to the transmission of diseases. During the 1920s until 1950s, thousands of Raccoon dogs were deliberately introduces to the eastern European countries from the Far East, in order to enrich the wild with this new valuable fur animal. The Raccoon dog is considered the most successful invading mammal in Europe, and in the last 20 years, it has invaded the western part of Denmark, namely Jutland. The Danish ministry of Environment reacted to the new threat by deciding to eradicate this species. In 2011, all animals shot and/or accidentally killed by traffic (N=70) were sent for post mortem analysis at the National Veterinary Institute. Concurrently, foxes originating from the same areas (N=60) were examined by *post mortem* analyses to compare helminth infections in the two species. Eight helminth species were isolated from both hosts; however, foxes harboured more helminth species per infected animal (average 3,1 helminth species/fox) than raccoon dogs (average 1,7 helminth species/raccoon dog). Prevalences of nematodes (*Uncinaria stenocephala*, *Toxocara canis* and *Toxascaris leonine*) and cestodes (*Mesocestoides sp.* and *Taenia spp.*) were significantly higher in foxes compared to that for raccoon dogs, while the latter had significantly higher prevalences of the two trematode species *Alaria spp.* and *Echinostomatidae*. Trematodes of the species *Cryptocotyle spp.* were equally prevalent in both of the hosts. No infections with *Echinococcus multilocularis* or *Trichinella spp.* were detected in any of the hosts. Morphologically, helminths of both hosts were identically with the exception of *Alaria* isolated from raccoon dogs which were highly abundant but significantly stunted in size. By comparing these results with those obtained from other countries, we can clearly see that raccoon dogs are not well established in Denmark. Helminths currently recovered from Danish raccoon dogs are mainly those that have direct life cycles or can be transmitted through amphibian or insect intermediate hosts, while those transmitted by rodents are less prevalent.

A pipeline for the production of human affinity-matured recombinant monoclonal antibodies to *Plasmodium falciparum.*

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In *Plasmodium falciparum*-endemic areas, clinical immunity in humans does develop and anti-malaria parasite antibodies have clearly been demonstrated to play a major role in this immunity. Recombinant *P. falciparum*-specific human monoclonal antibodies corresponding to those acquired by clinically immune individuals would be an important tool in epitope identification and in the verification of correct folding of recombinant produced *P. falciparum* proteins.
For this we are developing a new technology platform - a pipeline - for the production of human affinity-matured recombinant monoclonal antibodies (re-mAbs).

We immortalized and stimulated memory B cells *in vitro* from malaria-exposed donors to differentiate into antibody-secreting plasma cells. Supernatants were screened for antigen reactivity and cells from positive wells were single-cell-sorted. A multiplex RT-PCR followed by a multiplex PCR was performed, resulting in the amplification and fusion of the two expressed variable antibody genes from the light and the heavy chains, preserving the natural affinity matured light-heavy chain pairing. The resulting constructs were expressed in human HEK293 cells growing in suspension and the secreted re-mAbs purified. The obtained re-mAbs were tested for antigen specificity by ELISA or in Luminex multiplex assay, and further tested by flow cytometry for surface reactivity with *P. falciparum*-infected erythrocytes.

Our results confirm that the produced re-mAbs maintain the antigen specificity of the original memory B cell-derived antibody. The production of these re-mAbs can easily be scaled up, and at the present focus is on increasing the number of different re-mAbs processed.

Group a *Pfemp1* functional domains bind icam1 and induce cross-reactive and adhesion inhibitory antibodies during malaria infections.

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The *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) plays an important role in antigenic variation and pathogenesis of malaria. PfEMP1 proteins encoded by group A *var* genes appear to be involved in the pathogenesis of severe disease and have been suggested as attractive candidates for a vaccine against life-threatening *P. falciparum* malaria. In this study, we identified group A *pfd1235w*-like genes in Ghanaian isolates and found these to encode a three-domain cassette structure 64-80% identical to the equivalent region of *P. falciparum* clone 3D7 PFD1235w. Parasites expressing PFD1235w-like proteins on the surface of infected erythrocytes were found to mediate binding to ICAM1, a phenotype linked to cerebral malaria. ICAM1 binding was mediated by a particular sub-domain which induces cross-reactive and ICAM1 adhesion-inhibitory antibodies during *P. falciparum* infections. These results have implications for our understanding of how PfEMP1 interacts with host receptors and for the development of therapeutic interventions targeting ICAM1 binding malaria parasites.

Uncomplicated malaria associated with reversible hearing impairment in children.

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The extent of hearing impairment in children with malaria is unknown, and the evidence linking currently used artemisinin combination therapy with ototoxicity remains inconclusive. Audiometry was conducted in children aged 5 to 14 years with acute uncomplicated malaria, before treatment with artesunate+amodiaquine (n=37), artemether-lumefantrine (n=35), or amodiaquine (n=8), and repeated after 3, 7, and 28 days and 9-15 months. School children aged 5 to 14 years (n=57) living in the same area were included as control. The hearing threshold was significantly elevated in the acute illness stage compared with controls ($p<0.001$). The threshold elevations persisted one month after successful therapy, but hearing had normalised after 9-15 months. The hearing threshold of subjects treated with amodiaquine monotherapy continued rising during the first week of treatment compared with the other treatment groups ($p<0.001$). The persistence of the elevated hearing threshold levels for at least one month after an uncomplicated malaria attack may have potential implications for learning and cognitive development. The findings have implications for evaluating ototoxicity of antimalarial drugs.

POSTERS

A new system for higher recovery rate of water borne *Cryptosporidium* and *Giardia*.

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The two most common water borne pathogenic protozoa, *Cryptosporidium* and *Giardia*, are a common cause of outbreaks of diarrhea. Detecting these parasites in water samples depends on effective parasite recovery from the water matrix. The recovery rates of the currently used filter methods are low and the procedures are expensive and time consuming. These facts have motivated us to develop a new robust system for higher recovery of protozoan cysts from any fluid matrix. The new system consists of a sample collection chamber in which the fluid passes through a specially coated metallic filter with a carefully chosen pore size. On the reverse side of the filter a transducer delivers ultrasound waves in order to release trapped cysts and particles from the filter and break up parasite clusters. Purified *Cryptosporidium* oocysts and *Giardia* cysts were injected into the concentration unit and exposed to varying levels of ultrasonication. The concentrated parasite suspension was backwashed using air and the change in viability of the parasites was assessed by flow-cytometry. Without sonication, the recovery rate of *Cryptosporidium* was around 2%. After short term sonication for 5, 10 or 20 seconds, the recovery rates were stable and ranging from 75-83%. While sonication is usually used for cell lyses, it can be tuned into a useful tool for enhanced separation of retinates of parasites using a specially constructed filter unit and a sonication protocol. The filtration chamber further facilitated the concentration of parasites by ensuring a backwash volume of less than 1ml. The collected parasites may be used for molecular typing and other diagnostic and research purposes. The presented design of the filter system can be used as a robust, cheap, and time effective method of isolating water borne parasites in the laboratory.

The Duffy-Binding-Like β domain (DBLβ) of the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) variant, PFD1235w, binds ICAM1.

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Plasmodium falciparum is by far the most virulent human malaria parasite. *P. falciparum* variant erythrocyte surface antigens, known as PfEMP1, play a crucial role in malaria pathogenesis as they mediate adhesion to host endothelial receptors. The PfEMP1 variant, PFD1235w, encoded by the 3D7 group A *var* gene has been associated with severe malaria and erythrocytes infected with parasites expressing PFD1235w binds ICAM1. To identify the PFD1235w domain(s) responsible for ICAM1 binding we used recombinant protein (NTS, CIDR1□, DBL1□-CIDR1□, DBL□□domains, CIDR2□) and ICAM1 in Enzyme-Linked Immuno-Sorbent Assay (ELISA). We identified the DBLβ3-domain 4 (D4) of the PFD1235w to be responsible for ICAM1 binding in a concentration dependent manner and the binding could be inhibited by a panel of monoclonal ICAM1 antibodies. By using 3D protein modeling we generated different PfEMP1 hybrid molecules and truncated proteins in order to determine the essential binding region of the DBLβ3-D4 involved in the ICAM1 interaction. The hybrid molecules and truncated proteins were tested for ICAM1 binding in ELISA. Results indicate that the C-terminal of DBLβ3-D4 is directly involved in the ICAM1 interaction, while the N-terminal region is necessary for correct protein conformation. These results contribute to a greater understanding of how PfEMP1 interacts with endothelial receptors such as ICAM1 and provide a model for future analysis of other PfEMP1 variants adhering to ICAM1.

Plasmodium falciparum Erythrocyte Membrane Protein 1 Duffy Binding like-β Domains - optimization of expression and purification.

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The *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is accepted to be the primary adhesive protein responsible for cytoadherence of infected erythrocytes to post-capillary venules. These large multi-domain proteins are encoded by ~60 *var* genes per haploid genome, conferring a spectrum of adhesion phenotypes. Adhesion to host receptors is mediated by single or multi-domain interactions by constituent Duffy-Binding Like (DBL) domains, Cysteine-Rich Interdomains (CIDR), and the N-terminal Structure (NTS). Of particular interest are variants of the DBLβ domain, which have been demonstrated to bind to the host-receptor Intercellular Adhesion Molecule-1 (ICAM-1). Seeing as ICAM-1 binding has been associated with cerebral and severe malaria, there is significant interest in characterizing their binding interaction, as well as epitopes that may be targeted by neutralizing antibodies. In order to elucidate the structure of DBLβ domains at an atomistic level, recombinant His-tagged single-domain DBLβ proteins have been expressed in an *E. coli* system optimized for the formation of disulfide bonds. Following metal-ion affinity chromatography, the DBLβ domains were screened for ICAM-1 binding via ELISA. The DBLβ domains were then further purified using heparin-binding and ion exchange chromatography for further analysis. This body of work involves the optimization of expression and purification of single DBLβ domains in hopes to produce large quantities of correctly folded protein to be used for kinetic analysis and crystallographic studies. Seven DBLβ domains from six different parasite strains will be screened for their propensity to form crystals both as single domains and as co-crystals with ICAM-1, as well as have their respective affinities for ICAM-1 measured using Label-Free Surface Plasmon Resonance (LFSPR).

Ectoparasites on pigs and farmers' practices in relation to the infestations and diseases in Mbeya Region, Tanzania.

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A cross-sectional study was carried out from May to August 2011 in the Mbeya Region, Tanzania in order to describe the management practises and the ectoparasitic distribution and diversity within confinement and free range production systems of smallholder households. A total of 96 households practising confinement and 32 households practising free range were surveyed. The prevalence of ectoparasites on pigs in confinement and free range production systems were 24% [15-33] and 84% [71-91], respectively. Logistic regression models were designed to explore risk factors for the presence of ectoparasites. Keeping pigs in a free range system ($p<0.001$, OR=17.9 [4.0-76.1]) and the presence of neighbouring pigs ($p=0.018$, OR=4.33 [1.29-14.57]) were identified as risk factors for ectoparasites within both systems. Within the confinement system, contact with neighbouring pigs ($p=0.031$, OR=4.15 [1.14-15.1]) and the time interval since last treatment ($p=0.030$, OR=1.17 [1.02-1.35]) was identified as a risk factors. The prevalence of lice was 20% [12-28] in confined pigs and 63% [45-78] in free range pigs. Free ranging of pigs ($p=0.003$, OR=7.7 [2.0-30.0]) and presence of neighbouring pigs ($p=0.002$, OR=8.1 [2.2-30.6]) were identified as risk factors for the presence of lice. The prevalence of fleas was 5% and 13% within confined and free range, respectively. The prevalence of hard ticks among the free range pigs was 50%. Ectoparasites were found highly prevalent in both production systems, although more so in the free range production system. The smallholder farmers would benefit from more knowledge in regards to control and prevention of ectoparasites and on basic pig husbandry such as water and feeding requirements.

Multispectral direct detection of *trichinella* larvae on nylon filters.

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Although several new methods for *Trichinella* surveillance is being developed and risk based herd certification is a potential alternative to classical meat inspection, *Trichinella* testing by digestion assays are still widespread and considered the gold standard. A new vision-based method and instrumentation has been developed for the direct detection of *Trichinella* larvae recovered from a range of digestion processes used in classic meat inspection. The process allows for subsequent molecular analysis and thereby a thorough risk analysis of potential findings. The instrument consists of a table-top scanner unit and a PC which is running the application software. The user prepares the sample on a dedicated sample holder (35µm net), inserts the sample into the instrument and activates the software. The instrument then scans the entire surface of the net, capturing several multispectral images, which are analysed to form a combined analysis of the entire sample. The result of the analysis is displayed on the screen after a couple of minutes. For initial validation, *Trichinella* larvae were propagated in mice and released by artificial HCl–pepsin digestion. Known numbers were deposited on nylon filters. A close correlation between the manually verified number of *Trichinella* larvae recovered on a net and the number of pixels detected as "positive" by the instrument. The instrument was able to detect single larvae even in presence of muscle fibres. De-coiled larvae results in larger number of pixels, but over-estimation will not compromise consumer safety. No false negatives were found. Automation of the direct inspection methods may offer a cost effective method to certify meat free of *Trichinella* as compared to herd certification.

Real-Time Quantitative PCR Diagnoses of *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* at Danish Hospitals.

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It has been recently reported that *P. ovale* comprises two nonrecombining species, *P. ovale curtisi* and *P. ovale wallikeri*. Here, we report two Danish travellers who became ill after returning from Africa, one diagnosed as *P. ovale curtisi* and the other as *P. ovale wallikeri* by using a real-time quantitative PCR. The first patient had been travelling in Uganda, Kenya and Tanzania for 6 month before returning to Denmark in the autumn of 2009. The patient had been taken prophylactic doxycycline intermittently and was treated twice for malaria in Uganda. In January 2010, the patient had a relapse and was diagnosed as *P. malariae* by microscopic examination. The patient had a second relapse in October 2011. This time *P. ovale* was detected by microscopy and confirmed by real-time PCR. The second patient had been in Zambia for 2 months and developed fever seven days after returning to Denmark and stopping Doxycyclin prophylaxis. The aetiologic agent was diagnosed as *P. ovale* by microscopic examination, and this was also confirmed by real-time PCR. Both patients were negative in rapid tests using the BinaxNow Malaria Kit. By using specific primer-probe combination in real-time quantitative PCR, we were able to demonstrate that the first patient was infected with *P. ovale curtisi* and the second patient with *P. ovale wallikeri*. There is presently no evidence about difference in clinical pattern of malaria caused by the two parasite species but the identification to the species level is important to generate knowledge about this. Both our patients had a mild clinical course and were subsequently given eradication treatment with primaquine.

Imported Malaria 1994-2010, Hvidovre Hospital, Denmark.

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In Denmark we have observed a 75% decrease in number of imported malaria cases during the last decade. In this study, we describe clinical and epidemiological characteristics of patients with malaria seen at Department of Infectious Diseases (DID), Hvidovre Hospital, from 1994 to 2010.
Results: A total of 296 cases were included. 56% were male. Mean age was 34 years.
We observed a significant decrease in incidence from a mean of 20 imported cases/year the first 4 years observed, to a mean of 15 imported cases/year the last 4 years observed. The decreasing incidence observed, was primarily due to fewer imported cases from Asia, whereas imported cases from Africa remained stable.
We observed, that imported cases the first 8,5 years primarily consisted of ethnic Danes traveling as tourists, and work related travels, whereas the last 8,5 years imported cases was primarily ethnic Africans visiting friends and relatives.
68% (n=202) were diagnosed with a Plasmodium falciparum infection, 24% (n=71) with plasmodium vivax, 4% (n=13) with plasmodium ovale, 2% (n=5) with plasmodium malariae and 2% (n=6) with a double infection. 213 cases (72%) were imported from Africa, and 87% (n= 186) of those cases were plasmodium falciparum infections. 47 patients (16%) had severe malaria defined by WHO criteria. 39 (83%) of these cases were imported from Africa.
Conclusion: Imported malaria is decreasing. The risk of infection with the potentially severe plasmodium falciparum, is still significant when traveling to African countries. Patients at highest risk of imported malaria are African residents traveling to visit friends and family. Danish travelers seem to be more aware of the risk of malaria

The prevalence of selected endobiotic eukaryotes in different human cohorts.

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Background: Little is known about the significance of common intestinal parasites such as *Blastocystis* and *Dientamoeba* in health and disease. The prevalence of especially *Blastocystis* is significantly lower in patients with inflammatory bowel disease (IBD) than in patients with irritable bowel syndrome (IBS), non-IBD/non-IBS diarrhoea and healthy individuals. However, it is not known whether the prevalence of other endobiotic eukaryotes such as *Entamoeba* spp., *Enteromonas hominis*, *Pentatrichomonas hominis* and yeasts is also significantly lower in the IBD cohort compared to non-IBD cohorts.
Aim: To determine the prevalence and distribution of eukaryote organisms among patients with IBD and controls (IBS, patients with non IBS, and healthy), and to generate prevalence estimates of little studied protozoa such as *E. hominis* and *P. hominis.*
Methods: Faecal samples were collected from a total of 140 Danish individuals: 30 patients with IBD, 30 patients with *Dientamoeba fragilis* (non-IBD/non-IBS), 20 patients with IBS and *Blastocystis*, 20 patients with IBS without *Blastocystis*, 20 healthy patients with *Blastocystis*, and 20 healthy patients without *Blastocystis*. Samples were tested by PCR for *E. hominis*, *P. hominis*, *Entamoeba* spp. and yeast. Species identification was obtained by sequencing.
Results: The study is ongoing and results will be available in March 2012. We expect that the data will assist us in generating a hypothesis on the potential significance of endobiotic eukaryotes in patients with inflammatory bowel disease and irritable bowel syndrome.

Definition of humane endpoints and standardisation of a fatal malarial anaemia model.

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The survival in lethal models of malarial anaemia is not standardised and there is a need for definitions of humane endpoints used in such studies in order to comply with animal ethic demands. A low invasive infrared temperature measurement device was evaluated.
A/J mice were infected i.p. with either 10^4, 5×10^4, 10^5, 5×10^5, 10^6, or 5×10^6 Plasmodium *chabaudi* AS parasitized red blood cells (RBCs) with the aim of inducing illness ranging from relatively uncomplicated malaria with no fatalities to severe malaria with high rate of fatalities. Study parameters were mortality, haemoglobin, infected RBCs, reticulocytes, weight and temperature. The infrared temperature measurement was compared to rectal temperature measurement in both normal mice and mice terminal ill from P. *chabaudi* AS infection.
There was a strong relationship between mortality and parasite inoculums, with 0%, 40% 60%, 100%, 100%, 100% death respectively. Severe anemia was observed in all mice.
There was a close to perfect regression between rectal and IR measurement in both normal mice and mice terminal ill from P. *chabaudi* AS infection (slope: 1.0, r^2 0.98, intercept 0,0.54 and slope of 0.9, r^2 0,9374 and intercept 0,0.90) respectively.
In conclusion we found i.p. inoculation of $5x10^4$ to $1x10^5$ *P. chabaudi* AS in A/j mice a suitable model for fatal malarial anaemia, and we found a drop in body temperature to 30°C measured by detection of IR emission an unbiased humane endpoint for death in survival studies.

Molecular detection of natural Babesia bovis infection from water buffaloes (Bubalus bubalis) and crossbred cattle.

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Babesia bovis (*B. bovis*) is a major causative agent of bovine babesiosis, with a considerable worldwide impact. The objective of this study was to evaluate the usefulness of PCR assay and microscopical examination (ME) for detection of *B. bovis* in naturally infected and apparently healthy water buffaloes and crossbred cattle under field circumstances from Sharkia province of Egypt. A total 34 animals (20 crossbred cattle and 14 buffaloes) were clinically and laboratory investigated during the period from March to August 2008. Fifteen animals showed symptoms of bovine babesiosis while 19 animals were apparently healthy. Two blood samples were collected from each animal; one was used for preparation of Giemsa-stained smears for ME while the other sample was used for DNA extraction and PCR testing. Out of 34 cattle and buffaloes, ME identified 13 animals (38.2%) as infected by *B. bovis* whereas PCR identified 29 (85.3%). *B. bovis* infected animals showed high fever, anaemia, jaundice, haemoglobinuria, and accelerated heart and respiratory rates. Out of 15 animals clinically infected, PCR identified 14 animals (93.3%) as infected while ME identified only, 8 animals (53.3%). Out of 19 animals apparently healthy, 5 animals (26.3%) were identified as infected by ME meanwhile 15 animals (78.9%) were identified by PCR. In conclusion, our findings demonstrated that water buffalos are likely to have a natural tolerance to *B. bovis* pathogen and/or more likely to be persistent carriers which were not picked up by microscopy. The severity of clinical symptoms of *B. bovis* infection on water buffaloes was less than the severity of clinical symptoms appeared on cattle. PCR assay is more sensitive technique than microscopical examination for detection of *B. bovis* in both clinically infected and apparently.

Population dynamics and host reactions in young foxes following experimental infection with minute intestinal trematodes, *haplorchis* spp.

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Fishborne zoonotic trematodes (FZT) are highly prevalent in Southeast Asia and recent studies found that the most prevalent FZTs in both animals and humans were *Haplorchis* spp. Among the animal hosts dogs were found to have the highest intensity of infection and contribute the most to the contamination of the environment. Basic knowledge of the population dynamics and host reactions is needed to make recommendations for control of FZT. On this background, we conducted an experimental infection study with *Haplorchis* spp. using foxes as a model for dogs. Eight commercially bred foxes (5 months old), were each infected with 2000 metacercariae. Another three foxes were included as uninfected controls. Faecal examination for eggs was performed twice weekly using a sieving and sedimentation technique. The bodyweight was measured and standard haematological analysis was performed. Foxes were examined post-mortem for presence of adult FZT eight weeks post infection (p.i.). Following sectioning of the small intestine the predilection site of the flukes and the optimal method for obtaining the worms was determined. A decrease in appetite was observed in the infected foxes about ten days p.i. and a decline in body weight in the infected group was noticed in third week. Seven of eight infected foxes became faecal-positive for small trematode eggs at day nine p.i.. Faecal egg counts ranged between 0-95 eggs per g faeces (epg). Mean egg excretion after patency (day 9-55) was 10 epg. Adult flukes were present in the small intestine from all foxes at necropsy. To conclude, the study confirmed short prepatency, low egg excretion, establishment in all animals and location in the posterior end of jejunum. Further studies are warranted to explore more in depth specific host reactions. Our results provided new information on the animal hosts of *Haplorchis* spp. which can be used when providing recommendations for prevention and control of FZT.

Pfemp1 domains of different genomic origin share epitopes recognized by cross-reactive antibodies.

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Human malaria affects millions of people and is caused by 5 different species of *Plasmodium* parasites with *P. falciparum* causing the majority of the morbidity and mortality. The *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) antigens expressed by the parasite and transported to the surface of infected erythrocytes (IE) play a major role in antigenic variation, immunity to malaria and cytoadhesion of IE to different receptors such as ICAM1. PfEMP1 is a large protein family with a high degree of sequence variability both within individual genotypes and between different genotypes. Each PfEMP1 molecule consists of 4-9 domains of the two different domain types: Duffy-binding-like domain (DBL) and Cysteine-rich inter domain region (CIDR) which further classify into different subtypes. The exact composition of the different PfEMP1 molecules is not completely random as some specific domains group together to form cassette families which can be found in many different genomes. The PFD1235w cassette family has been associated with severe disease in children and presence of antibodies towards parts of the cassette has been found to confer protection against malarial fever.

In this study we tested for cross-reactivity between 53 domains of PfEMP1 from different parasite genomes. This was done by competition ELISA using a human plasma pool of 147 individuals from a malaria endemic area in Tanzania.

The results showed broad cross-reactivity between the different heterologous DBL2b domains of the PFD1235w cassette family. The remaining DBL and CIDR domains not belonging to the PFD1235w cassette family only showed little or no cross-reactivity in line with previous similar studies.

Isolate- and intraerythrocytic age-specific changes in knob characteristics of *plasmodium falciparum*-infected erythrocytes expressing the Pfemp1 protein VAR2CSA.

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The virulence of *P. falciparum* malaria is related to the parasite's ability to evade host immunity through clonal antigenic variation and tissue-specific adhesion of infected erythrocytes (IEs). The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family expressed on electron-dense knobs on the IE surface is central to both. Differences in receptor specificity and affinity of expressed PfEMP1 are important for IE adhesiveness, but it is not known whether differences in the number and size of the knobs on which PfEMP1 proteins are located also play a role.
We used atomic force microscopy (AFM) to characterize knobs on the surface of *P. falciparum*-infected erythrocytes. Ten *P. falciparum* isolates selected *in vitro* for expression of a particular PfEMP1 protein (VAR2CSA) were examined. Knob density increased from early to late trophozoite stage in all isolates, and varied among the isolates. There was no significant relationship between knob density and IE adhesiveness.

Our data show that knob density varies among *P. falciparum* isolates expressing the same PfEMP1 protein (VAR2CSA). It is tempting to speculate that this reflects differential isolate-specific strategies to balance IE sequestration and evasion of host immunity.

Prevalence of lymphatic filariasis in an endemic district of Nepal.

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Lymphatic filariasis (LF) caused by *Wuchereria bancrofti* is widespread and a major public health problem in Nepal. It is endemic in 60 out of 75 districts and around 90% (25 million) population lives in areas with risk of transmission. Despite the huge problem, very limited studies were conducted regarding the epidemiology of LF. This cross sectional study was carried out at Bardiya district, one of the endemic districts of Nepal. A total of 510 randomly selected individuals aged 5 years and above were screened for circulating filarial antigen (CFA) in finger prick blood by Immuno-Chromatographic Test (ICT) card. It is a highly sensitive diagnostic tool that determines the presence of adult filarial worms in the lymphatic system. One hundred twenty-seven out of 510 (24.9 %) were found to be positive for CFA. Prevalence of CFA was found almost equal in both males (48%) and females (52%). Further, prevalence of CFA was determined in all age groups; including 5-10 years (5%), 11-20 years (40%), 21-30 years (16%), 31-40 years (13%), 41-50 years (10%) and above 50 years (16%). The present study was done to obtain baseline data on the prevalence of CFA which may be used as a background for implementing and monitoring the national LF elimination programme, including mass drug administration.

Resuspension and settling of helminth eggs in water: interactions with cohesive sediments.

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Helminth parasite eggs in low quality water represent main food safety and health hazards and are therefore important indicators used to determine whether such water can be used for irrigation. When helminth eggs are removed from water by sedimentation they accumulate in the sediment. Resuspension of deposited helminth eggs in e.g. irrigation canals will lead to increased concentration of suspended eggs in the water. Our study aimed to determine the erodibility (erosion rate and erosion threshold) and settling velocity of *Ascaris* and *Trichuris* eggs as well as cohesive sediment at different time points after incorporation into the sediment. Cohesive sediment collected from a freshwater stream was used to prepare a sediment bed onto which helminth eggs were allowed to settle. The erodibility of both sediment and helminth eggs was found to decrease over time indicating that sediment consolidation takes place along with incorporation of eggs into the sediment. This was supported by finding of a higher settling velocity for eggs associated with particles as compared to eggs in clean water. The incorporation into the sediment bed decrease the mobility of helminth eggs and the aggregation increases the effective settling velocity of the eggs leading to reduced mobility. Our findings documents that helminth eggs should not be viewed as single entities in water systems when modeling the distribution of eggs since both erodibility and settling velocity of eggs are determined by mobility of the sediment present in the water stream. Recalculation of the erosion threshold for helminth eggs and sediment showed that even at relatively low current velocities i.e. 0.07-0.12 m s^{-1} the eggs will demonstrate high mobility in open irrigation channels. These environmental factors affecting resuspension must be taken into account when developing models for sedimentation of helminth eggs in different water systems.

Using production of three full full-length recombinant Pfemp1 proteins as a tool for the molecular dissection involved in rosetting, a cytoadhesion phenotype and virulence factor of the malaria parasite *Plasmodium falciparum*.

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The pathogenicity of the apicomplexan parasite *Plasmodium falciparum*, which is responsible for most of malaria-related severe morbidity and essentially all the mortality, is related to the capacity of *P. falciparum*-infected erythrocytes (IEs) to adhere to vascular host receptors. This adhesion is mediated by a family of clonally variant *P. falciparum* erythrocyte membrane proteins called PfEMP1. *P. falciparum*-IEs display multiple adhesive phenotypes, including rosetting, which is the adhesion of uninfected erythrocytes to IEs. Expression of PfEMP1 proteins causing rosettes to form has repeatedly been implicated in the pathogenesis of severe *P. falciparum* malaria.

Here we report the production of recombinant proteins representing three full-length PfEMP1 proteins involved in rosetting. All the three *Baculovirus*-infected insect cell-produced proteins appear to be correctly folded, as they bind non-specific IgM and specific IgG as predicted. Correctly folded full-length protein has been instrumental in dissecting the molecular details of the adhesive function of the VAR2CSA protein mediating IE adhesion in the placenta. VAR2CSA is the only other recombinant PfEMP1 reported in the literature so far.
In addition to the full-length proteins, we have also produced 23 single- and double-domain recombinant constructs of the same PfEMP1 proteins, to further assist our analysis of their adhesive properties.