Assessing malaria drug resistance in US military areas of operation using microarrays.

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Background:
Malaria inflicts an incredible burden on the health of the human population worldwide with 250-500 million cases per year and approximately one million deaths annually, predominately in children under five(1,2). While eradicated in parts of the developed world, roughly 40% of the world's population live in areas where malaria is endemic(1,3). In addition to increasing morbidity and mortality, malaria significantly decreases productivity and economic growth in endemic regions(3).

On account of the current worldwide geopolitical situation, the United States military is conducting active operations in areas around the world where malaria is endemic. This situation creates additional hazards for US military personnel in foreign countries where malaria is endemic and forces the US military to utilize extensive malaria prophylaxis. Current pharmaceuticals are effective at controlling malaria, but drug resistance is increasingly on the rise(4). Recently, the first treatment failure, although not specific parasite resistance, has been reported for artemisinin-based combination therapy(5) which is the current gold standard for malaria treatment underscoring the need for new drugs and an effective vaccine. In addition to new treatment methods, increased surveillance of *Plasmodium* parasites, the causative agent of malaria, needs to be undertaken in endemic areas so emerging resistant strains can be identified early and appropriate public health measures be instituted to limit their spread.

During the Med-into-Grad clinical rotation, three cases of malaria in San Diego county were presented by an Infectious Disease fellow from the United States Navy (USN) at the city-wide Infectious Disease Case Conference. In all three cases the patients had been infected while in a foreign country endemic for malaria, two from Africa and one from Afghanistan. The two patients who had recently traveled to Africa were infected with *Plasmodium flaciparum*, the most deadly form of malaria, and had not taken malaria prophylaxis. The third case occurred four months after the patient returned from active duty in Afghanistan and was caused by *Plasmodium vivax*. *P. vivax* has the unique ability to form hypnozoites, which are dormant forms of the parasite in the patient's liver and are resistant to normal malaria drugs. Primaquine can be used to eradicate these dormant liver stages and is standard for all US military personnel returning from Afghanistan, where 90% of the malaria cases are caused by *P. vivax*. The patient in this case, though, did not receive primaquine treatment upon return to the US.

The three malaria cases presented during the Med-into-Grad clinical rotation demonstrate the opportunity to conduct malaria research with the US military in a mutually beneficial collaboration. Recently our lab has demonstrated the ability to detect all single nucleotide polymorphisms (SNP), insertion/deletions (in/dels), and copy number variations (CNV) present between three laboratory isolates of *P. flaciparum* using a custom whole genome tiling microarray(6). Through identification of these genetic differences, we can identify those parasites that are resistant to current drugs, or, conversely, identify the mechanism of resistance in those parasites that have been proven to be resistant to current anti-malarials. By obtaining malaria samples from the

US military and conducting our microarray analysis, we can provide the US military with an accurate picture of the specific malaria threat that they face in each of their specific areas of operations. Additionally, by amassing a library of genetic differences of field isolates, we can contribute substantially to the understanding of the genetics behind malaria drug resistance along with identifying the genetic basis of other parasite specific biological processes, such as hypnozoite formation.

Specific Aim:
The specific aim of this short proposal is to utilize clinical samples and data amassed by the US military from its operations in malaria endemic regions to identify emerging resistant parasites as well as broaden our understanding of the genetic basis of the parasites unique biology.

Obtain malaria field samples from the US military. As malaria has been eradicated from the US for 50 years, malaria research in the US is dependant on a few *in vitro* culture adapted isolates or field samples from endemic regions. We will work with the US military to design a protocol to obtain samples from all patients, both US military personnel and foreign nationals, that are treated for malaria by US military doctors. This protocol will consist of obtaining five milliliters of blood from a patient with malaria confirmed through a thick or thin blood smear. Blood will be obtained immediately after diagnosis and before treatment. We have experience with a similar protocol for samples collected in Iquitos, Peru

Manipulation and storage of samples. After we obtain the samples we will isolate the parasites from the patient's blood. If the patient was infected with *P. falciparum* we will attempt to grow the parasites *in vitro* as an established protocol for *in vitro* culture of this *Plasmodium* species exists(7). If the patient was infected with another *Plasmodium* species, the samples will be stored appropriately until microarray analysis. Which *Plasmodium* spp. the patient is infected with will be determined by thin blood smear analysis or a commercial diagnostic method such as Paracheck Pf. (Orchid Biomedical Systems).

DNA sample preparation and microarray analysis. DNA will be isolated and prepared using our standard protocol as described previously(6). For those *P. falciparum* strains that are unable to be adapted to culture and the samples from patients infected with a *Plasmodium* species other than *P. falciparum*, sample DNA will be amplified using GenomePlex Complete Whole Genome Amplification (Sigma).

DNA will be hybridized to either our *P. falciparum* or our *P. vivax* (manuscript in preparation) whole genome tiling microarray using standard protocol and analyzed as recently described(6). We will look specifically for those genetic variations responsible for drug resistance initially. In this way we can provide a dynamic picture of the current drug susceptibilities of the specific malaria parasites that are present in US military area of operations. Later after additional clinical data has been acquired, including treatment outcome, we can run additional analysis on the microarray data to identify genetic variations leading to new drug resistance and genes that are required for unique aspects of parasite biology.

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