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Succinctus

Travellers as sentinels: Assaying the worldwide distribution of polymorphisms associated with artemisinin combination therapy resistance in *Plasmodium falciparum* using malaria cases imported into Scotland

Carol W. Hunja [a,b], Holger Unger [d,f,1], Pedro E. Ferreira [e], Richard Lumsden [c], Sheila Morris [f], Rashid Aman [b], Claire Alexander [g], Toshihiro Mita [h,i,1], Richard Culleton [a,*,1]

[a] Malaria Unit, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan
[b] Centre for Research in Therapeutic Sciences (CREATES), Strathmore University, Nairobi, Kenya
[c] Haematology Laboratory, Western General Hospital, NHS Lothian Crewe Road, Edinburgh, Scotland, United Kingdom
[d] Department of Medicine (Royal Melbourne Hospital), The University of Melbourne, Melbourne, Australia
[e] Department of Microbiology, Tumour and Cell Biology, Karolinska Institutet, Stockholm, Sweden
[f] Regional Infectious Diseases Unit, NHS Lothian, Western General Hospital, Crewe Road, Edinburgh, Scotland, United Kingdom
[g] Scottish Parasite Diagnostic and Reference Laboratory, Greater Glasgow & Clyde NHS, House on the Hill, Stobhill Hospital, Glasgow, Scotland, United Kingdom
[h] Department of Molecular and Cellular Parasitology, Juntendo University School of Medicine, Tokyo, Japan
[i] Department of International Affairs and Tropical Medicine, Tokyo Women's Medical University School of Medicine, Tokyo, Japan

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ABSTRACT

There is growing evidence that *Plasmodium falciparum* parasites in southeastern Asia have developed resistance to artemisinin combination therapy. The resistance phenotype has recently been shown to be associated with four single nucleotide polymorphisms in the parasite's genome. We assessed the prevalence of two of these single nucleotide polymorphisms in *P. falciparum* parasites imported into Scotland between 2009 and 2012, and in additional field samples from six countries in southeastern Asia. We analysed 28 samples from 11 African countries, and 25 samples from nine countries in Asia/southeastern Asia/Oceania. Single nucleotide polymorphisms associated with artemisinin combination therapy resistance were not observed outside Thailand and Cambodia.

Artemisinin combination therapies (ACTs) are the first line treatment for *Plasmodium falciparum* malaria in most endemic countries of the world. *Plasmodium falciparum* readily develops resistance to antimalarial drugs (Petersen et al., 2011), and the loss of artemisinin efficacy due to resistance would constitute a global health disaster (Fairhurst et al., 2012). Recently, the spectre of ACT resistance has emerged in southeastern Asia, specifically in Thailand and Cambodia (Noedl et al., 2008; Dondorp et al., 2009); the same area in which parasites resistant to chloroquine (CQ) and sulfadoxine-pyrimethamine (S/P) first emerged (Mita et al., 2011). There is a very real danger that ACT resistance will spread outside southeastern Asia, as happened with resistance to CQ and S/P, although containment strategies are currently being developed (Dondorp et al., 2010).

Of paramount importance in the monitoring of the spread of drug resistance is the availability of molecular markers of resistance, such as the well-characterised mutations in the chloroquine resistance transporter gene (*crt*) and in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes for CQ and S/P resistance, respectively. Such markers allow the surveillance of the prevalence of resistance in areas where clinical resistance might be masked by host immunity, and facilitate dissection of the geographical spread of drug-resistant parasites.

Resistance to ACTs is characterised by delayed clearance of parasites following drug treatment. Following the first reports of parasites with delayed clearance rates, it was shown that the resistance phenotype was likely to have an underlying genetic component (Anderson et al., 2010). This implied that genetic mutations had arisen in a subset of parasites in western Cambodia that decreased their sensitivity to ACTs and that these mutations were being selected by ACT pressure in this region. It also raised the possibility of identifying the genes responsible for ACT resistance, which would allow both the dissection of the mode of action of

* Corresponding author. Address: Malaria Unit, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Tel.: +81 95 8197903; fax: +81 95 8197805.
E-mail address: richard@nagasaki-u.ac.jp (R. Culleton).
[1] These authors contributed equally to this work.

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ACT and its resistance, and the development of molecular resistance-surveillance approaches. A genomic region associated with the resistance phenotype was, indeed, described in 2012 (Cheeseman et al., 2012), rapidly followed by the identification of four single nucleotide polymorphisms (SNPs) on chromosomes 10, 13 and 14, which appear to be linked to resistance (Takala-Harrison et al., 2013). Two of these SNPs, MAL10-688956 (A) and MAL13-1718319 (T) were proposed to be suitable molecular markers for delayed parasite clearance following ACT resistance. Although these SNPs are not thought to confer resistance themselves, and despite the fact that they have been identified in some regions before ACT resistance was reported in southeastern Asia, they could be linked to the actual genetic drivers of resistance, which could exist in parasite populations that have never been exposed to ACTs and which would be selected for when ACT pressure is applied to the population. It is important, therefore, to assess the worldwide prevalences of these markers, in order to generate baseline data on the probability of ACT resistance arising in various regions of the world. Of particular concern, of course, is Africa, where the majority of the deaths attributed to *P. falciparum* malaria occur and where the consequences of ACT resistance would be felt most severely.

Travellers returning to non-endemic countries with *P. falciparum* malaria offer a unique and valuable source of parasite DNA for molecular surveillance studies. If there are sufficient numbers of travellers, then samples can be collected from a wide geographic range and all can be processed using the same protocols and in the same laboratories under the same conditions, allowing for more reliable inter-region comparisons to be made. In contrast to samples collected in endemic countries, travellers returning to their home countries with malaria often have complete and thorough medical histories, often have no immunity to the parasite through previous exposure, have good records of anti-malarial drug usage and can usually identify both the region in which the parasite was acquired and the time at which exposure occurred.

This study provides a proof of principle for the use of travellers' malaria in studies of the worldwide prevalence of markers associated with drug resistance in *P. falciparum*. A total of 27 *P. falciparum* samples representing 11 (mainly African) countries of origin were obtained from cases of travellers' malaria imported into Scotland between 2009 and 2012. As a further objective of this work was to assay the global prevalence of the SNP markers associated with ACT resistance, an additional 24 samples representing a further eight countries mainly in southeastern Asia and Oceania were obtained from field studies conducted previously. Sample origins are shown in Fig. 1.

For the Scottish imported malaria cases, samples were collected at the haematology laboratory of the Western General Hospital (WGH) in Edinburgh, where malaria diagnosis for all patients who present, or are referred, to the adjacent Regional Infectious Diseases Unit (RIDU) is performed, and in Glasgow, where samples were collected at the Scottish Parasite Diagnostic and Reference Laboratory (SPDRL) at Stobhill Hospital, Glasgow. SPDRL receives samples from across Scotland from patients suspected of, or diagnosed with, malaria, and undertakes confirmatory testing. Samples that were sent from Edinburgh to SDPRL but had already been collected at the Edinburgh site were excluded from collection at SPDRL.

As part of routine clinical management, 3–5 ml of venous blood was collected from patients with suspected malaria into an EDTA vacutainer. Blood samples were subsequently sent to respective laboratories for routine full blood count examination, blood-smear microscopy (thick and thin smear) and malaria rapid diagnostic test card examination. For those patients positive for malaria by microscopy and/or rapid test, and for whom sufficient blood sam-

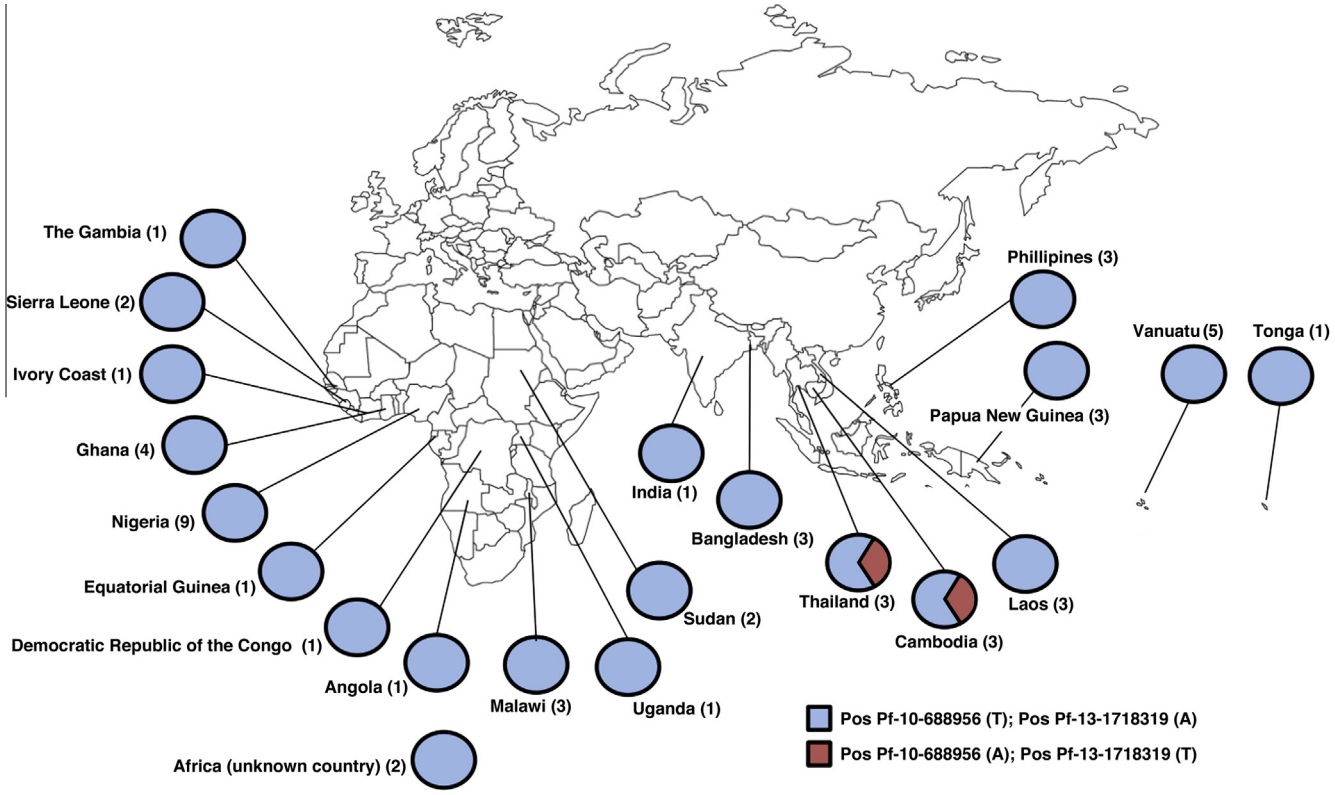

Fig. 1. Geographic origin (and number) of the *Plasmodium falciparum* samples used in this study. Circles indicate the proportion of parasites from each region with single nucleotide polymorphism MAL10-688956 (T) and MAL13-1718319 (A) genotypes (blue), or with MAL10-688956 (A) and MAL13-1718319 (T) genotypes (red), associated with delayed parasite clearance rates following artemisinin combination therapy.