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Flashback to the 1960s: Utility of archived sera to explore the origin and evolution of *Plasmodium falciparum* chloroquine resistance in the Pacific

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Abstract

The increasing frequencies of *Plasmodium falciparum* strains that are resistant to chloroquine (CQ) and other antimalarials are resulting in a global resurgence of malaria morbidity and mortality. CQ resistance (CQR) is associated with multiple mutations in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene. The mode and tempo of the accumulation of substitutions leading to these complex CQR haplotypes remain speculative due to the dearth of samples temporally spanning the evolution of drug resistance. The origin and evolution of the CQR alleles of Papua New Guinea (PNG) is particularly ambiguous. It remains unclear whether the *pfcrt* haplotype in PNG resulted from an independent origin of a CQR haplotype identical in sequence to the South American haplotype, or if this haplotype originated in South America and recombined into a Southeast Asian-derived genome.

We sequenced a segment of *pfcrt* exon 2 from 398 plasmid clones derived from archival human sera collected in the Pacific before and after the first reported cases of CQ treatment failure ($n = 251$) and modern samples ($n = 147$). None of the 251 *pfcrt* plasmid clones from nine archival samples displayed the C72S or the K76T mutations that are characteristic of CQR strains. In contrast, these two amino acid substitutions were present in all 147 *pfcrt* plasmid clones from five samples collected between 2001 and 2003; thus, the archival samples represent the baseline parasite genetic diversity before the evolution of CQR strains.

We are currently expanding our analyses to include additional samples from the series described here and from series collected in the 1970s and the 1980s to evaluate the geographic origin of CQR strains in the Pacific and the validity of the sequential point mutation accumulation model of CQR evolution.

Keywords: Chloroquine resistance; Pacific; *pfcrt*; Archival samples

1. Introduction

The development and global distribution of chloroquine (CQ) as an inexpensive and relatively safe antimalarial was one of the most significant public health interventions of the 20th Century (Wellems and Plowe,

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2001). The resurgence of malaria is in large part the result of increasing treatment failures associated with the evolution of drug resistance, particularly CQ resistance (CQR) (Wernsdorfer and Noedl, 2003). Almost 500 million cases and 1–3 million deaths are attributed to malaria each year (Wellems and Miller, 2003). Of the four malaria parasite species that affect humans, *Plasmodium falciparum* is the most debilitating. Outside of sub-Saharan Africa, lowland Papua New Guinea (PNG) is the only region where *P. falciparum* malaria infections reach holoendemic levels (Mehlotra et al., 2002; Muller et al., 2003). Due to its low cost and few side effects, CQ had been the first line of treatment for uncomplicated malaria worldwide since the late 1940s. Within 20 years of its initial introduction, however, the first cases of CQ treatment failure for *falciparum* malaria appeared independently in Southeast Asia (Thai-Cambodian border) and South America (Colombia and Peru), followed by cases in New Guinea in the mid-1970s and East Africa by 1977 (Payne, 1987; Wootten et al., 2002). Widespread CQ treatment failure has forced most countries to replace CQ with the combination of sulfadoxine and pyrimethamine (SP) as the first line treatment against *falciparum* malaria (Farooq and Mahajan, 2004). As resistance to SP becomes increasingly problematic, artemisinin-based combination therapies (ACTs) are likely to become the new standard for those countries that can afford them (Mutabingwa, 2005).

Genetic polymorphisms in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) are crucial in conferring CQR (Wellems and Plowe, 2001; Wongsrichanalai et al., 2002). Among the 10 amino acid substitutions in *pfcrt* that have been observed in CQR parasite isolates (Chen et al., 2003), the K76T mutation has been shown to be the most closely associated with CQR (Djimde et al., 2001). While the K76T substitution was found globally, distinct *pfcrt* haplotypes associated with CQR were observed in parasite isolates from Southeast Asia (residues 72–76: CVIET) and South America (residues 72–76: SVMNT), supporting at least two independent origins of CQR (Payne, 1987; Fidock et al., 2000). Despite their geographic proximity to Southeast Asia, PNG parasites displayed CQR-associated *pfcrt* haplotypes identical to those from South America (Mehlotra et al., 2001; Nagesha et al., 2003). In contrast, chromosome-wide microsatellite data revealed a greater genetic affinity of PNG parasites to those from Southeast Asia rather than those from South America (Anderson et al., 2000). Whether the discrepancy between these two sets of genetic markers indicates an independent origin of identical CQR haplotypes in both PNG and South America or the recombination of the South American *pfcrt* haplotypes into a Southeast Asian-derived parasite genome, remains unresolved (Mehlotra et al., 2001).

The mechanistic evolution of CQR *pfcrt* haplotypes also remains unresolved. Hastings et al. (2002) suggested that the acquisition of CQR occurred via a sequential accumulation of mutations. Parasite strains bearing the first mutations are thought to better tolerate CQ at subtherapeutic levels, thus allowing for earlier re-infection relative to strains without these mutations. According to this model, the CQR-associated K76T mutation represents the final and critical step that occurred on the background of these slightly advantageous, tolerance conferring mutations (Hastings et al., 2002). The validity of this model has not been adequately evaluated due to the absence of both parasites bearing intermediate haplotypes and archival samples collected prior to the evolution of CQR (e.g. Mehlotra et al., 2005).

These two unresolved questions, the origin and the order of accumulation of the multiple substitutions of CQR haplotypes can both be addressed by examining *pfcrt* haplotypes of parasites collected in the Pacific during the evolution of drug resistance over the past 50 years. To our knowledge, no study examining the *pfcrt* haplotype in parasites from PNG prior to the first cases of CQ treatment failure in 1976 (Yung and Bennett, 1976) has been conducted. In this preliminary study, we explored the feasibility of analyzing the genetic variation of *P. falciparum* collected in Melanesia during the 1960s–1980s to gain insight into the evolution of the complex *pfcrt* CQR haplotype currently common in the Pacific. Additional contemporary samples from East Sepik Province, PNG were included for geographically controlled comparisons.

2. Materials and methods

The archival human sera were selected from the NIH-NINDS-LCNSS Serum Archive of Binghamton University, New York. The sera examined consisted of the following samples: Sepik, Wosera (years 1960–1963; $n = 15$), Sepik, Wewak (1965; $n = 15$), Sepik, Wewak (1970; $n = 10$), Central New Britain (1956; $n = 10$), Vanuatu (formerly New Hebrides) (1963; $n = 10$), Vanuatu (1972; $n = 20$), and the Solomons Islands (1987–1989; $n = 15$). In addition, five individuals described in a previous study (Mita et al., 2006) with microscopically confirmed *falciparum* malaria from East Sepik Province collected in 2001–2003 were also included (Fig. 1).

DNA was extracted from archival human sera using the QIAamp DNA Blood Mini Kit (QIAGEN Biosciences, Germantown, MD) according to the manufacturer's instructions. For the 1963 Vanuatu samples, DNA