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Microarray-based comparative genomic analyses of the human malaria parasite *Plasmodium falciparum* using Affymetrix arrays

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Abstract

Microarray-based comparative genomic hybridization (CGH) provides a powerful tool for whole genome analyses and the rapid detection of genomic variation that underlies virulence and disease. In the field of *Plasmodium* research, many of the parasite genomes that one might wish to study in a high throughput manner are not laboratory clones, but clinical isolates. One of the key limitations to the use of clinical samples in CGH, however, is the miniscule amounts of genomic DNA available. Here we describe the successful application of multiple displacement amplification (MDA), a non-PCR-based amplification method that exhibits clear advantages over all other currently available methods. Using MDA, CGH was performed on a panel of NF54 and IT/FCR3 clones, identifying previously published deletions on chromosomes 2 and 9 as well as polymorphism in genes associated with disease pathology.

Keywords: *Plasmodium falciparum*; Chromosomal deletions; Whole genome amplification; Comparative genomic hybridization

1. Introduction

Malaria inflicts a serious health and economic burden on those countries in which it is endemic. The most severe form of this disease is caused by *Plasmodium falciparum*, responsible for between 300 million and 500 million clinical cases and between 1 million and 3 million deaths annually, most of these in young children in sub-Saharan Africa [1]. The impact of this disease serves to highlight the importance of developing new programmes of mosquito vector control and anti-parasitic therapy to replace those rendered ineffectual by the spread of drug resistance. A longer-term vaccine-based strategy has met with some limited success, although the development of a cheap efficacious vaccine suitable for Sub-Saharan Africa is still some distance off [2].

The complete annotated *P. falciparum* genome sequence was published in 2002 [3] with subsequent gene expression [4,5] and proteomic analyses [6,7] providing valuable insights into potential new intervention targets. Comparative genomic hybridization (CGH) provides a complementary approach to the identification of potential vaccine and drug targets. Using microarray technology, genomic DNA from one isolate/clone can be compared against an arrayed reference genome and differences between the two identified. In cancer research, such technology is widely used not only for the identification of cancer-related genes [8,9] but also to detect DNA copy number aberrations or loss of heterozygosity (LOH) [10]. In the investigation of infectious disease, CGH has assessed vaccine response heterogeneity for *Mycobacterium* sp. [11], investigated

Abbreviations: CGH, comparative genomic hybridization; DOP-PCR, degenerate oligonucleotide primed PCR; KAHRP, knob-associated histidine-rich protein; LMP, ligation mediated PCR; MDA, multiple displacement amplification; PCR, polymerase chain reaction; PEP, primer extension preamplification; PfEMP3, *Plasmodium falciparum* erythrocyte membrane protein 3; WGA, whole genome amplification

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host–pathogen relationships [12,13] and using long oligonucleotide microarrays, has catalogued the degree of genetic variation between laboratory lines of *P. falciparum* [5].

Comparative genomic analyses have also played an important role in the annotation of *Plasmodium* sp. genomes. Using the complete genome sequences of *P. falciparum* and *P. yoelii*, comparative annotation of several other *Plasmodium* sp. genomes are under way (see http://www.genedb.org/). New initiatives to sequence a second laboratory isolate of *P. falciparum*, as well as clinical *P. falciparum* isolates, has increased the power with which we can assess genomic differences between these parasites. Perhaps the best-studied variations are those within the sub-telomeric regions, where members of the multigene families involved in antigen variation and cytoadhesion (namely *var*, *rifin*, and *stevor*) are located [14–17]. These exhibit a high degree of polymorphism between strains [18,19]. Microarray-based CGH is a simple and efficient investigative tool to analyse variation and polymorphism, not only within these gene families, but also on a genome-wide scale.

One limitation of CGH is the quantity of genomic DNA required for the microarray hybridization. Analysis of small numbers of cells, such as those from clinical samples, requires whole genome amplification (WGA) to derive the necessary assay material. WGA procedures such as primer extension preamplification (PEP) [20], degenerate oligonucleotide primed PCR (DOP-PCR) [21] and ligation-mediated PCR (LMP) [22] have all been developed as reliable approaches for DNA amplification (as reviewed in [23]). PEP and DOP-PCR procedures were specifically designed for the analysis of single cells or very small DNA samples. However, WGA techniques face some limitations. Imbalanced amplification of microsatellite [24] and SNP (small/single nucleotide polymorphism) alleles [25,26], incomplete coverage of the genome in the amplification products [27], as well as the introduction of artificial sequence variation, have been observed. In an attempt to overcome the problems associated with the use of degenerate primers binding to multiple sites in these amplification techniques, LMP was developed. Ligation of an adapter to genomic DNA fragments prior to PCR amplification requires only a single primer in the subsequent PCR, thus reducing the complexity of the amplification protocol. Furthermore, as an alternative to PCR-based WGA protocols, an isothermal procedure for rolling circle amplification of DNA templates, using the DNA polymerase from the bacteriophage Φ29 [28], has been adapted as an amplification method for linear templates [27]. Isothermal multiple displacement amplification (MDA) uses random hexamers containing phosphorothioate-modified nucleotides as primers, and relies on the high processivity, fidelity and strand displacement ability of the Φ29 DNA polymerase [29,30]. Quantitative real time PCR analysis indicates that MDA-based WGA generates a high quality product, with the amplified DNA products essentially indistinguishable from that of unamplified genomic DNA [27,31].

Here we compare two WGA techniques, LMP and MDA, using microarray-based CGH to assess their suitability for the analysis of the extremely AT-rich *P. falciparum* genome. Using small amounts of input DNA to mimic likely yields from clinical samples, we have compared several *P. falciparum* isolates and laboratory clones. In doing so, we have been able to unequivocally identify genomic differences of likely biological significance. The protocol we have developed thus lends itself to the investigation of sequence polymorphism in the pathology of clinical malaria.

2. Materials and methods

2.1. P. falciparum culture and genomic DNA extraction

P. falciparum clones and isolates were cultured in vitro in donated human type O erythrocytes as described [32]. The reference clone 3D7, used in the *P. falciparum* genome sequencing project [3] and thus in the design of the scrMalaria Affymetrix microarray (see Section 2.5), is derived from the isolate NF54. The Ituxi (IT) isolate, genetically indistinguishable from many FCR3 laboratory lines [33], has been selected for binding to ICAM-1 [34] to derive the common laboratory clone A4. The P1B5 clone was derived from A4 parasites selected for adhesion to uninfected erythrocytes [35]. Limiting dilution of P1B5 gave rise to the isogenic clones 1B12, 1H2, 2A11, 1H10, 2H3, 3D9 and 1F5 used in this study [36]. Genomic DNA was isolated and stored as described [37].

2.2. Ligation-mediated PCR (LMP)

The LMP protocol used has been adapted from that of Tangavelu and Bankier (personal communication) and [10,38]. Genomic DNA was restricted using either *Dpn*II, *Apo*I or *Dpn*II and *Apo*I for 10 min at 37 °C before being heat-inactivated for 30 min at 65 °C. Appropriate linkers were added and annealed for 20 min at 65 °C followed by a temperature ramp down to 15 °C at 1 °C/min. Ligation was then initiated by adding 5 units of T4 DNA ligase (NEB) and 1 mM of rATP to the reaction mixture and incubated for 22–24 h at 15 °C.

The primary amplification of linker-ligated restriction fragments was achieved by PCR, using 4.46 units of Expand Long Polymerase (Roche) in the 1× PCR buffer provided with dNTPs (0.4 mM each, biotin-labelled dCTP was added when direct-labelling of PCR products employed) using the program: one cycle at 94 °C for 4 min then 38 cycles of (94 °C for 40 s, 55 °C for 30 s, 68 °C for 1 min 45 s), finishing with 68 °C for 5 min. PCR products were purified using Qiagen columns as recommended by the manufacturer.

2.3. Multiple displacement amplification (MDA)

Reagents used were supplied with the GenomiPhi DNA Amplification Kit (GE Healthcare). Up to 80 ng of genomic DNA in 1 μl was added to 9 μl of sample buffer (50 mM Tris–HCl pH 8.2, 0.5 mM EDTA containing random hexamer primers) and denatured at 95 °C for 3 min. Φ29 DNA polymerase mix, including additional random hexamers, was mixed on ice with 9 μl of reaction buffer containing dNTPs and the mixture added to the denatured DNA sample. The MDA reaction was allowed to proceed for 18 h at 30 °C. The enzyme