The threat of drug-resistant malaria – trends and applicability of molecular markers of drug resistance

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Malaria control in Sub-Saharan Africa (SSA)

Success in control of *P. falciparum* in SSA mainly due to:

Implementation of interventions/measures:
- Preventive measures (ITNs, Indoor residual spraying (IRS))
- Improved diagnostics (RDTs)

Efficacious drugs to treat malaria:
- *Artemisinin-based combination Therapies (ACTs)*
  - No drug resistance in SSA

Preventive drug treatment:
  - Intermittent preventive treatment of infants (IPTi),
  - IPT of pregnant women (IPTp)
  - Children (seasonal malaria chemoprevention (SMC))

Major threat against the control of malaria in SSA: drug resistance
Malaria control – the challenge of drug resistance

Learning from history; resistance will always emerge

Chloroquine
Pyrimethamine/sulfadoxine (Fansidar®) - SP


A continuous need to monitor drug resistance
Malaria control – Identifying resistance

- *In vivo* drug efficacy trials and *In vitro* drug susceptibility tests

- Molecular makers of drug resistance: SNPs in *Pf* genes => resistance to antimalarial drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Resistance gene</th>
<th>Main SNPs (aa at position)</th>
<th>NB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td><em>Pfcrt</em></td>
<td>72-76 CVMNK (WT)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72-76 CVIET (MT)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72-76 SVMNT (MT)</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td><em>Pfdhfr</em></td>
<td>N51I</td>
<td>High P resistance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C59R</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>S108N</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>51+59+108 = 3 MT</td>
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</tr>
<tr>
<td>Sulfadoxine</td>
<td><em>Pfdhps</em></td>
<td>A437G</td>
<td>High SP resistance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K540E</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>437+540 = 2 MT (+ <em>Pfdhfr</em>=&gt; 5MT)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A581G</td>
<td>Super SP resistance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+581 = 3 MT (+<em>Pfdhfr</em>=&gt; 6MT)</td>
<td></td>
</tr>
</tbody>
</table>

Evolution of SP resistance: 108+51/59 MT in *Pfdhfr* then 437, 437+540, 437+540+581 MT in *Pfdhps*
Molecular markers of drug resistance to guide policy - IPTi

If we consecutively can map markers of drug resistance =>

Surveillance of drug resistance:

• Change in prevalence of mutations causing resistance over time =>

• Change in drug policy

  (if you have a drug to change to...)

• Examples: SP (IPTi and IPTp) and CQ
The use of molecular markers of drug resistance

- Often molecular markers have merely confirmed that there is resistance *in vivo*:

- Prevalence of 540E (high SP resistance) confirms that there is high SP resistance *in vivo* in East Africa

(Sampled at various time points (low prev. = earlier studies))

www.drugresistancemaps.org
Molecular markers of drug resistance to guide policy - IPTi

SP is still recommended for IPTi (as policy: only Burkina Faso)

- IPTi: provide effective protection against multiple infections and etc.  

- However, in countries such as Tanzania with high level of SP resistance, no protective effect  

  - As a result of high prevalence of 5MT in Pfdhfr and Pfdhps (measured as high prevalence of 540 MT)

- Threshold for when to use IPTi; based on molecular data:

  - WHO recommend only to use SP for IPTi

  "...where parasite resistance to SP is not high – defined as a prevalence of the Pfdhps 540 mutation of ≤ 50%.”

http://www.who.int/malaria/news/WHO_policy_recommendation_IPTi_032010.pdf?ua=1
Thresholds for protective efficacy of IPTi

Pfdhps- 540E

www.drugresistancemaps.org
Molecular markers of drug resistance to guide policy - IPTp

SP is used as IPT for pregnant women (IPTp)

- IPTp-SP for all pregnant women at each scheduled antenatal care visit until the time of delivery (3 times, one month apart)

- IPTp-SP prevents: placental infection, clinical malaria, maternal and fetal anaemia, low birth weight and neonatal mortality


- IPTp seems effective even in areas with high prev. of 540 MT; however, what if an additional mutation emerged?
Prevalence of \textit{Pfdhps} haplotypes in Korogwe, Tanzania –

Is IPTp still effective in areas where we now have the additional MT? (now; 6MT in \textit{Pfdhfr} and \textit{Pfdhps})

Alifrangis et al. (2009), \textit{Am J Trop Med Hyg}, 80(4), 2009
The use of molecular markers of drug resistance - IPTp

STOPPAM – Strategies to Prevent Pregnancy Associated Malaria

- Cohort of pregnant women living in Korogwe, NE-Tanzania

- 995 women included, all on IPTp
- Low prevalence of malaria; Among the women completing follow-up, 76 had a total of 96 episodes of malaria.

- Prevalence of 540 MT = 100%, 581 MT = 54%

Molecular markers of drug resistance to guide policy - IPT

2MT vs. 3MT infections =>
mean reduction of birthweight of 326g


"Consider discontinuing IPTp-SP when the population prevalence of...mutation 540E is greater than 95%, AND the prevalence of mutation 581G is greater than 10%, as it is likely to be ineffective"

Molecular markers of drug resistance to guide policy - IPT

Kavishe et al. Malar J (2016) 15:335
Molecular markers of drug resistance to guide policy - CQ

Can molecular markers be used to measure increased sensitivity of *P. falciparum* to abandoned drugs, e.g. Chloroquine (CQ)?

Ex. In Tanzania; CQ was abandoned in 2001 (officially)

- Tanga, NE-Tanzania
- >95% prev. Of CQ resistant parasites
- Now..

(Unpublished data)
Molecular markers of drug resistance to guide policy - CQ

<table>
<thead>
<tr>
<th>Region</th>
<th>Prev. Of CVMNK Wildtype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tanga</td>
<td>93.2</td>
</tr>
<tr>
<td>Coastal</td>
<td>93.5</td>
</tr>
<tr>
<td>Mtwarra</td>
<td>93.2</td>
</tr>
<tr>
<td>Kagara</td>
<td>85.7</td>
</tr>
<tr>
<td>Mwanza</td>
<td>88.4</td>
</tr>
<tr>
<td>Mbeya</td>
<td>92.7</td>
</tr>
<tr>
<td>Overall</td>
<td>91.0</td>
</tr>
</tbody>
</table>

Across Tanzania: >85% of parasites are wildtypes and thus, susceptible to CQ

Mohammed et al., 2013 Malaria J: 12:415
Molecular markers of drug resistance to guide policy - ACT

A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria

Frédéric Arjey1,2, Benoit Witkowski1, Chanaki Amaratunga4, Johann Béghain1,2, Anne-Claire Langlois1,2, Nimol Khim3, Saorin Kim5, Valentine Duru5, Christiane Bouchier6, Laurence Ma7, Pharath Lim1,4,7, Rithea Leang8, Socheat Duong8, Sokunthea Sereng9, Seila Suo10, Char Meng Chhong10, Denis Mey Boun11, Sandle Ménard10, William O. Rogers12, Blaise Genton12, Thierry Fandeur12, Olivo Miotto1,2,3, Pascal Ringwald14, Jacques Le Bras15, Antoine Berry14, Jean-Christophe Barale1,2, Rick M. Fairhurst14, Françoise Benoit-Vical16,17, Odile Mercereau-Puijalon1,2,8 & Didier Ménard14

Resistance to artesunate => K13-propeller polymorphisms

- A marker of ACT resistance *before* ACT resistance has been reported in SSA
- However, we do not know which SNPs will be important in SSA
**Molecular markers of drug resistance to guide policy - ACT**

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<td>High SP resistance Super SP resistance</td>
</tr>
<tr>
<td>Various antimalarials</td>
<td>Pfmdr1</td>
<td>N86Y, Y184F, S1034C, 1048, D1246N</td>
<td></td>
</tr>
<tr>
<td>Artemisinins</td>
<td>K13</td>
<td>Various</td>
<td></td>
</tr>
</tbody>
</table>
Determining molecular markers of drug resistance

PCR followed by various methodologies for SNP detection

- RFLP (enzymes cut to distinguish either WT or MT)
- Allele specific PCR
- Sequence specific oligonucleotide probe – ELISA

K13...sequencing is necessary:

- Sanger sequencing
- NGS...
  - (DTU Profs Ole Lund/Frank Aarestrup)
Determining molecular markers of drug resistance using NGS

Figure 3. Amplicon design

PF3D7_0417200 | bifunctional dihydrofolate reductase thymidilate synthase (DHFR-TS) | CDS length = 1827

PF3D7_0523000 | multidrug resistance protein (MDR1) | CDS length = 4260

PF3D7_0609000 | chloroquine resistance transporter (CRT) | CDS length = 1275

PF3D7_0810800 | hydroxymethylidihydropterin pyrophosphate dehydrogenase-dihydropyroate synthase (PPLK-DHPS) | CDS length = 2121

PF3D7_1343700 | kelch protein, putative (K13) | CDS length = 2181
Determining molecular markers of drug resistance using NGS

1. Gene specific PCR:

PCR products of:

- **Multiplex 1**
  - Mdr1 (1)
  - K13 (1)
  - K13 (3)
  - K13 (5)

- **Multiplex 2**
  - Dhfr (1)
  - K13 (4)

- **Multiplex 3**
  - Mdr (8)
  - Dhps (3)
  - K13 (3)

- **Simplex**
  - Crt 2

Non-annealing (not dhfr-related overhang) (will be incorporated into PCR products)
Determining molecular markers of drug resistance using NGS

2. Index PCR (necessary to link a Miseq sequence to a individual sample)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
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</thead>
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<tr>
<td>Index_1_F</td>
<td>AATGATACGGCGACCAACGAGATCTACACaaaagggaCGTCGCGCAGCGT</td>
</tr>
</tbody>
</table>

Anneal to overhangs
From gene PCR

Index 8bp Adapter (bind to flow cell of Miseq)

Indexing primers:
- Anneal to the overhangs
- Contain overhangs consisting of individual 8-base indices
- Adapter sequences that will allow the final PCR product to bind to the flow cell.

Indexing 8bp sequence:
- 50 variants in forward primers
- 50 variants in reverse primers
- 2450 combinations!

Sidsel Nag, unpublished
Determining molecular markers of drug resistance using NGS

Index PCR: using unique combinations of 8bp primers for each individual sample (consisting of fragments of all target genes) Samples are...Indexed/barcoded

PCR product:

Pooling of Index PCRs

Miseq

Sidsel Nag, unpublished
Determining molecular markers of drug resistance using NGS

<table>
<thead>
<tr>
<th>#CHROM</th>
<th>POS</th>
<th>ID</th>
<th>REF - DHPs</th>
<th>ALT</th>
<th>QUAL</th>
<th>GB1-10C</th>
<th>GB1-10D</th>
<th>GB1-10E</th>
<th>GB1-10F</th>
<th>GB1-10G</th>
<th>GB1-10H</th>
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<td>0/0:457:0</td>
<td>0/0:542:0</td>
<td>1/1:2672:255:0</td>
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<tr>
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</tr>
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</tr>
</tbody>
</table>

Sidsel Nag, unpublished
For at ændre "Enhedens navn" og "Sted og dato":
Klik i menulinjen, vælg "Indsæt" > "Sidehoved / Sidefod".
Indføj "Sted og dato" i feltet for dato og "Enhedens navn" i Sidefod.

Byt billede:
Ny slide og klik på ikon, indsæt billede.