Recent Experience of Investigational Parasite Detection Methods in Controlled Human Malaria Infection Studies

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Disclosures

- Biofire Defense (consulting)
- Novartis (clinical trial support)
Outline

- *Plasmodium* 18S rRNA/rDNA
- Characteristics of NATs at major CHMI sites
- NAT biomarker kinetics in different CHMI study designs
- NAT-based rescue treatment thresholds
- Appropriate sampling frequency
- Recrudescence vs. gametocytemia
NAT-based approaches accelerate infection detection.

Seattle Biomed MC-001 Demo Trial by Pf 18S rRNA qRT-PCR assay

Detection of *Plasmodium* nucleic acids

- Extraction from whole blood
  - DNA *or* RNA *or* Total nucleic acid
- Reverse transcription or cDNA synth *(if applicable)*
- DNase treatment *(if pfs25/pvs25 testing)*
- Real-time PCR *(usually with hydrolysis/Taqman probes)*
  - Quantitative *or* Qualitative
  - Most assays target *Plasmodium* 18S rRNA/rDNA
    - A few non-CHMI labs target multi-copy non-18S rRNA targets or mitochondrial cytochrome oxidase subunit 1, etc.
**Plasmodium** 18S rRNA/rDNA is the most common molecular biomarker in CHMI studies.


*Sequestered* (undetectable +/−)

'Circulating' (detectable)

Pf 18S rRNA/rDNA

rDNA: 2 A-type and 2 S-type
rRNA: 3,500-10,000 A-type rRNAs

*P. falciparum* as shown

Pv/Po/Pm/Pk do not sequester

Gametocytes also express 18S rRNA (not shown)

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*P. falciparum* 18S rRNA/rDNA NATs are positive 2-5 days earlier than TBS.

Primary endpoint in Seattle
Performance characteristics of NATs at major CHMI sites
# Plasmodium 18S rRNA/rDNA NATs

<table>
<thead>
<tr>
<th>CHMI site</th>
<th>18S target</th>
<th>Extraction</th>
<th>Analytical Sensitivity (est. para/mL)</th>
<th>Volume (µL)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>UW/Seattle MCTC</td>
<td>A</td>
<td>Abbott m2000 sp</td>
<td>20</td>
<td>50 (into LB)</td>
<td>1</td>
</tr>
<tr>
<td>U. Maryland</td>
<td>S</td>
<td>Manual QIAamp DNA Mini Kit</td>
<td>20</td>
<td>500</td>
<td>2</td>
</tr>
<tr>
<td>VRC/NIH Clin Ctr</td>
<td>A</td>
<td>bioMerieux easyMag</td>
<td>500</td>
<td>200 (into LB)</td>
<td>3</td>
</tr>
<tr>
<td>Jenner/Oxford</td>
<td>S</td>
<td>Qiagen QIAsymphony</td>
<td>10</td>
<td>500 (filtered)</td>
<td>4</td>
</tr>
<tr>
<td>RUMC/Nijmegen</td>
<td>S</td>
<td>Roche MagNA Pure</td>
<td>20</td>
<td>500</td>
<td>5</td>
</tr>
<tr>
<td>QIPD/QIMR/Brisbane</td>
<td>S</td>
<td>Manual QIAamp DNA Mini kit</td>
<td>64</td>
<td>250 (pRBC)</td>
<td>6</td>
</tr>
<tr>
<td>Tübingen</td>
<td>A/S</td>
<td>Qiagen QIAsymphony</td>
<td>5</td>
<td>500</td>
<td>7</td>
</tr>
</tbody>
</table>

**References:**

A, A-type 18S rRNA; S, S-type 18S rRNA; LB, bioMerieux NucliSENS lysis buffer; Prim, primary; Sec, secondary

Summary data current as of June 29, 2016
EQA sample results were plotted ($\log_{10}$ parasites/mL) for participating laboratories (bars = mean +/- 95% confidence interval). Nominal (expected) values were High (300,000 parasites/mL); Mid (6,000 parasites/mL); Low (600 parasites/mL); Very Low (60 parasites/mL), Trace (6 parasites/mL) and Negative (no parasites; ). Samples with no parasites detected were plotted as 0.1 $\log_{10}$ parasites/mL. *Lab 2 quantities were generated by regression of $C_T$ values to expected EQA values and are provided to visualize variation and qualitative agreement. Full data with the exception of Lab 6 data are available in Murphy et al. 2014. PLoS One 9(5): e97398.
NAT biomarker kinetics in different CHMI study designs
CHMI by different routes, different stages?

• Mosquito bite vs. intravenous sporozoites:
  – Does not change the duration of LS
  – No indication for NAT testing on Days 0-5
  – No difference in NAT kinetics

• Sporozoite vs. iRBC challenge:
  – NAT positivity depends on assay LoD and parasite density.
  – Sporozoite challenge: sufficient iRBCs usually present by Day 7-8
  – iRBC challenge: sufficient iRBCs usually present by Day 4
## LoD Considerations

**ROUTE 1:** 5 mosquitoes

- (# sporozoites/mosquito)
- % of sporozoites injected

**ROUTE 2:**

- 3500 spz by DVI
- % that successfully invade
- \(~3 \times 10^4\) merozoites/hepatocyte x # of infected hepatocytes = Max. # iRBCs upon emergence

**ROUTE 3:**

- Infected RBCs by needle inoculation
- \(1.8 \times 10^3\) iRBCs

**Positive result if NAT LoD is 10-100 est. para/mL.**

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### Table

<table>
<thead>
<tr>
<th>Day (spz)</th>
<th>Tot. Para./Total RBCs</th>
<th>= Est. para/mL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-6</td>
<td>Undetectable (10 iHep)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>6.5</td>
<td>(3 \times 10^5/2.25 \times 10^{13})</td>
<td>60 (0.000001%)</td>
</tr>
<tr>
<td>8.5</td>
<td>(3 \times 10^6/2.25 \times 10^{13})</td>
<td>600 (0.00001%)</td>
</tr>
<tr>
<td>10.5</td>
<td>(3 \times 10^7/2.25 \times 10^{13})</td>
<td>6000 (0.0001%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day (iRBC)</th>
<th>Tot. Para./Total RBCs</th>
<th>= Est. para/mL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(1.8 \times 10^3/2.25 \times 10^{13})</td>
<td>&lt;1 (0.000000007%)</td>
</tr>
<tr>
<td>2</td>
<td>(1.8 \times 10^4/2.25 \times 10^{13})</td>
<td>3.6 (0.00000007%)</td>
</tr>
<tr>
<td>4</td>
<td>(1.8 \times 10^5/2.25 \times 10^{13})</td>
<td>36 (0.0000007%)</td>
</tr>
<tr>
<td>6</td>
<td>(1.8 \times 10^6/2.25 \times 10^{13})</td>
<td>360 (0.000007%)</td>
</tr>
<tr>
<td>8</td>
<td>(1.8 \times 10^7/2.25 \times 10^{13})</td>
<td>3600 (0.00007%)</td>
</tr>
</tbody>
</table>

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CHMI using TBS-based rescue

**Sporozoite inoculum**

- Inoculum: 5 mosquito bites or 3500 DVI
- Time to positivity if naïve/no drug treatment:
  - NAT(+) Usually by D7-8
  - TBS(+) Usually by D10-13
  - Rescue Rx Upon TBS positivity

**iRBC inoculum**

- Inoculum: 1.8x10^3 P. falciparum-infected RBCs
- Time to positivity if naïve/no drug treatment:
  - NAT(+) Usually by D4
  - TBS(+) Usually by D9-11
  - Rescue Rx Upon TBS positivity
CHMI using NAT-based rescue

Sporozoite inoculum

iRBC inoculum

Inoculum: 5 mosquito bites or 3500 DVI

1.8x10^3 P. falciparum-infected RBCs

Time to positivity if naïve/no drug treatment:

<table>
<thead>
<tr>
<th></th>
<th>NAT(+)</th>
<th>TBS(+)</th>
<th>Rescue Rx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum</td>
<td>Usually by D7-8</td>
<td>Usually TBS-negative</td>
<td>Usually by D8-10</td>
</tr>
<tr>
<td>NAT LoD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Usually by D4</td>
<td>Usually TBS-negative</td>
<td>Usually by Day 7-9</td>
<td></td>
</tr>
</tbody>
</table>
Study-specific NAT rescue thresholds

**PROPHYLAXIS** at liver and/or RBC stage
- Goal: Trigger rescue treatment; minimize symptoms
- Rescue threshold range ~100-500 est. p/mL
  - Seattle MCTC/UW: 250 est. para/mL (daily testing)
  - RUMC/Nijmegen: 100 est. para/mL (daily testing)
  - VRC: Two positive results (LoD ~500 est. para/mL)
  - Some centers still rely on TBS for safety endpoint (>2x10^4/mL)

**RADICAL CURE** at RBC stage
- Goal: generate safe/adequate RBC-stage infection, initiate experimental dosing and monitor for clearance/recrudescence
- Higher threshold than for liver stage prophylactic studies
  - QIMR: 800-1,000 est. para/mL (Pasay et al. 2016 *JID* doi: 10.1093/infdis/jiw128)
Time (days) from positive NAT at the indicated threshold (x-axes) to malaria-related symptoms (A) or TBS positivity (B). Example data from once daily testing in a closed Seattle-based study. ‘Any positive’ indicates all positives including unquantifiable low positive results. Symptoms include all protocol-defined malaria-associated symptoms including headache, fever, chills, abdominal pain, myalgia, low back pain and nausea. TBS served as the primary study endpoint. Red bars, mean +/- 95%CI.
# Rescue treatment threshold modeling

<table>
<thead>
<tr>
<th>p values for Thresholds compared to:</th>
<th>TBS positivity</th>
<th>Any symptom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any positive RT-PCR including Low Positives</td>
<td>****</td>
<td>****</td>
</tr>
<tr>
<td>First positive RT-PCR ≥20 p/mL</td>
<td>****</td>
<td>****</td>
</tr>
<tr>
<td>First positive RT-PCR ≥250 p/mL</td>
<td>****</td>
<td>**</td>
</tr>
<tr>
<td>First positive RT-PCR ≥500 p/mL</td>
<td>****</td>
<td>0.06</td>
</tr>
<tr>
<td>Two positive RT-PCR including one ≥250 p/mL</td>
<td>***</td>
<td>0.28</td>
</tr>
<tr>
<td>First positive RT-PCR ≥1,000 p/mL</td>
<td>**</td>
<td>0.73</td>
</tr>
<tr>
<td>First positive RT-PCR ≥10,000 p/mL</td>
<td>0.99</td>
<td>0.52</td>
</tr>
<tr>
<td>TBS positive</td>
<td>NA</td>
<td>0.13</td>
</tr>
<tr>
<td>Malaria-related symptom onset</td>
<td>0.13</td>
<td>NA</td>
</tr>
</tbody>
</table>

**** p < 0.0001; *** p < 0.001; ** p < 0.01; * p < 0.05 (one-way ANOVA)  
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What is the appropriate sampling frequency?
Daily testing is suitable for infection detection...

Infection detection post-sporozoite CHMI

Models that estimate liver burden rely on the maximum estimated density on the first day of positivity.

Some centers increase sampling on D7-8.

Left: Seattle MCTC/CIDR, Right: example model calculations based on Hermsen (2004 AJTMH)
Biomarker clearance is rapid following rescue treatment with FDA-approved drugs (atovaquone-proguanil shown).

20 subjects, Seattle MCTC
More frequent sampling is used for modeling clearance in experimental radical cure studies.

FIG 1 Effect of lag phase and tail exclusion on the calculation of the clearance rate constant. (Modified from Flegg et al. with permission of the author.)

Modeling radical cure in IBSM

Parasitemia/ml

-500 mg
-750 mg
-1,000 mg
-600/300 mg (CQ)

Parasitemia/ml

-5 mg/kg
-10 mg/kg
-15 mg/kg

Parasitemia in mefloquine treated, *Plasmodium*-infected volunteers (this study) or malaria patients. Black lines: this study (mefloquine). Blue lines: patients treated in earlier studies with mefloquine, Red lines: chloroquine (CQ: 600/300 mg) (28, 29).

Recrudescence vs. gametocytemia
Piperaquine Monotherapy of Drug-Susceptible *Plasmodium falciparum* Infection Results in Rapid Clearance of Parasitemia but Is Followed by the Appearance of Gametocytemia

Cielo J. Pasay, Rebecca Rockett, Silvana Sekuloski, Paul Griffin, Louise Marquart, Christopher Peatey, Claire Y. T. Wang, Peter O’Rourke, Suzanne Elliott, Mark Baker, Jörg J. Möhrle, and James S. McCarthy

1QIMR Berghofer Medical Research Institute, 2School of Chemistry and Molecular Biosciences, 3School of Medicine, University of Queensland, 4Queensland Pediatric Infectious Diseases Laboratory, 5Q-Pharm, 6Department of Infectious Diseases, Mater Health Services and Mater Research Institute, and 7Australian Army Malaria Institute, Brisbane; 8Medicaments pour Tous, Rolle, and 9Medicines for Malaria Venture, Geneva, Switzerland

Pasay et al. 2016. *JID* 214:105
NAT-based differentiation between gametocytemia & recrudescence

- *Plasmodium* 18S rRNA/rDNA (positive in asexual and gametocyte stages)
- Gametocyte-specific mRNA (e.g., Pfs25, Pfs230 and Pvs25 or other mRNA targets)

**Example A**

**Example B**

Pasay et al JID 2016 (Slide provided by J. McCarthy - QIMR)
Standards, Calibrators & EQA

• Standards (run controls)
  – Infected whole blood (no commercial source)

• Calibrators
  – Plasmids encoding full-length (A or S) (several labs) or hybrid plasmid (5’S + 3’A-type 18S rRNA genes) (UW)
  – Full-length Pf 18S rRNA as custom Armored RNA (UW)

• External quality assurance
  – WHO EQA scheme for malaria NAT in development
Summary

• Most common target: *Pf* 18S rRNA/rDNA
• NATs in use at most CHMI centers with increasing use for primary safety and/or efficacy endpoints
• Useful in sporozoite and iRBC CHMI with rescue thresholds as major difference
• Ongoing issues: recrudescence vs. gametocytes; harmonization; standards; calibrators; EQA
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     • Kevin Zhou
   – Robert Coombs
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   – CFAR Retrovirology Core

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   Emma Fritzen
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Top: Photo by Peter Ginter, New York Times, 3/4/08