Molecular diagnosis of malaria

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Putative advantages of molecular diagnosis of malaria

- correct (unequivocal?) identification of species
- increased sensitivity
- identification of mixed infections
- quantitative (if realtime format is used)

yet... you spent 2 hours on a malaria diagnosis practical without DNA tests
What do we expect of a malaria test?

•

•

•
Technical aspects

• original purpose of test
  – scale and time to result
  – quality control
• false positives: contamination
  – nested PCR assays
  – open systems/closed systems
  – lab set-up
• false negatives: inhibition
  – DNA extraction
  – internal control
Conventional PCR

- ~2 hours amplification
- ~1 hour running time
- 5 minutes visualization

Realtime PCR

- ~1 hour amplification & detection
Technical aspects

• original purpose of test
  – scale
  – quality control

• false positives: contamination
  – nested PCR assays
  – open systems/closed systems
  – lab set-up

• false negatives: inhibition
  – DNA extraction
  – internal control
quality control

• **UK NEQAS** (UK Nat’l External Quality Assessment Service)
• **QCMD** (Quality Control for Molecular Diagnostics)
• **INSTAND e.V.** (Society for Promotion of Quality Assurance in the Medical Laboratories)

*no*

international quality assurance scheme for molecular diagnosis of malaria
• Only quantification, no species

This study represents an evaluation of the first commercially available standardized real-time PCR assay for the diagnosis of malaria. Our results indicate that the RealArt Malaria LC PCR assay is rapid (assay time, <45 min), sensitive (99.5%), and specific (100%) for the detection of malaria parasites in febrile returned travelers.
Technical aspects

• original purpose of test
  – scale
  – quality control

• false positives: contamination
  – nested PCR assays
  – open systems/closed systems
  – lab set-up

• false negatives: inhibition
  – DNA extraction
  – internal control
nested PCR: DNA extraction – primary amplification – secondary amplification

closed system: tube is discarded unopened after analyses

open system: tubes opened for analysis

reagent preparation → amplification → amplification & analysis
Technical aspects

• original purpose of test
  – scale
  – quality control
• false positives: contamination
  – nested PCR assays
  – open systems/closed systems
  – lab set-up
• false negatives: inhibition
  – DNA extraction
  – internal control
sample collection on filters:
easy storage,
but more prone to contamination and human error

manual extraction:
easy for small numbers, but more prone to contamination and human error

automated extraction:
less contamination during extraction, but only cost-effective for large numbers
PCR for malaria

- epidemiology
  - in mosquito
  - in man

- primary diagnosis
  - saliva/urine
  - blood

- confirmatory diagnosis
  - mixed infections
this PCR seems too sensitive for clinical purposes in an endemic population.
PCR for malaria

• epidemiology
  – in man
  – in mosquito
• primary diagnosis
  – saliva/urine
  – blood
• confirmatory diagnosis
  – mixed infections
Quantitative Detection of *Plasmodium falciparum* DNA in Saliva, Blood, and Urine

Table 3. Diagnostic accuracy of nested polymerase chain reaction (PCR) assays and independent microscopy.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Positive predictive value, %</th>
<th>Negative predictive value, %</th>
<th>(\kappa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microscopist 1</td>
<td>96 (87–100)</td>
<td>99 (97–100)</td>
<td>91 (80–97)</td>
<td>99 (98–100)</td>
<td>0.92 (0.87–0.98)</td>
</tr>
<tr>
<td>Microscopist 2</td>
<td>98 (90–100)</td>
<td>96 (94–98)</td>
<td>81 (69–90)</td>
<td>100 (98–100)</td>
<td>0.87 (0.79–0.94)</td>
</tr>
<tr>
<td>Nested PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>98 (90–100)</td>
<td>95 (92–97)</td>
<td>74 (61–83)</td>
<td>100 (98–100)</td>
<td>0.81 (0.73–0.89)</td>
</tr>
<tr>
<td>Saliva</td>
<td>73 (58–84)</td>
<td>97 (95–99)</td>
<td>79 (64–89)</td>
<td>96 (93–98)</td>
<td>0.72 (0.61–0.83)</td>
</tr>
<tr>
<td>Urine</td>
<td>32 (20–47)</td>
<td>98 (96–99)</td>
<td>73 (50–89)</td>
<td>91 (87–93)</td>
<td>0.4 (0.25–0.54)</td>
</tr>
</tbody>
</table>

**NOTE.** Consensus expert microscopy, as defined in table 2, was used as the reference standard. CI, confidence interval.
Only limited evaluation in clinical import-malaria settings

Morassin AJTMH 2002: all patients in 1 year in Toulouse
An average of 2.48 PCR-based diagnoses daily

### Table 1

<table>
<thead>
<tr>
<th>Type of PCR result</th>
<th>Positive QBC result</th>
<th>Negative QBC result</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive <em>P.spp</em> or <em>Pf</em></td>
<td>104</td>
<td>32</td>
<td>136</td>
</tr>
<tr>
<td>Negative <em>P.spp</em> or <em>Pf</em></td>
<td>0</td>
<td>393</td>
<td>393</td>
</tr>
<tr>
<td>Total</td>
<td>104</td>
<td>425</td>
<td>529</td>
</tr>
</tbody>
</table>

*Pspp = Plasmodium species; Pf = P. falciparum.

32 discrepancies:
14 (previously) under treatment
10 (semi-)immune immigrants
5 travelers under chemoprofylaxis (1 treated)
3 unknown
Conclusions

• Morassin AJTMH 2002:
• “The PCR-based diagnosis of malaria appeared to be a useful tool that was suitable as a second-line method when the results of conventional techniques were negative in patients presenting a syndrome consistent with malaria, as well as yielding an accurate species identification.”
PCR for malaria

- epidemiology
  - in man
  - in mosquito
- primary diagnosis
  - saliva/urine
  - blood
- confirmatory diagnosis
  - mixed infections
Multiplex real-time PCR for the diagnosis of malaria: correlation with microscopy

L. Dormond¹, K. Jaton-Ogay², S. de Vallière², B. Genton², J. Bille¹,³ and G. Greub¹,³

<table>
<thead>
<tr>
<th>PCR</th>
<th>Results of thin smear microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmodium falciparum (n = 75)</td>
<td>Plasmodium falciparum and P. malariae (n = 3)</td>
</tr>
<tr>
<td></td>
<td>Plasmodium falciparum (n = 67)</td>
</tr>
<tr>
<td></td>
<td>Negative microscopy (n = 5)</td>
</tr>
<tr>
<td>Plasmodium vivax (n = 6)</td>
<td>Plasmodium vivax (n = 5)</td>
</tr>
<tr>
<td></td>
<td>Negative microscopy (n = 1)</td>
</tr>
<tr>
<td>Plasmodium malariae (n = 1)</td>
<td>Plasmodium malariae (n = 1)</td>
</tr>
<tr>
<td>Plasmodium falciparum and</td>
<td>Plasmodium falciparum (n = 1)</td>
</tr>
<tr>
<td>Plasmodium ovale (n = 3)</td>
<td>Plasmodium vivax (n = 1)</td>
</tr>
<tr>
<td>Plasmodium falciparum and</td>
<td>Plasmodium falciparum and P. vivax (n = 1)</td>
</tr>
<tr>
<td>Plasmodium malariae (n = 2)</td>
<td>Plasmodium falciparum (n = 2)</td>
</tr>
<tr>
<td>Plasmodium falciparum and</td>
<td>Plasmodium falciparum (n = 1)</td>
</tr>
<tr>
<td>Plasmodium malariae and Plasmodium ovale</td>
<td>Plasmodium falciparum (n = 1)</td>
</tr>
<tr>
<td>PCR negative</td>
<td>Plasmodium falciparum (n = 1)</td>
</tr>
</tbody>
</table>
correlation between quantitative PCR and quantitation by microscopy

Log of DNA copies/mL as determined by PCR

Log of parasites/mL as derived from microscopy

- Parasitaemia > 500’000 parasites/mL
- Parasitaemia ≤ 500’000 parasites/mL

$y = 0.9126x$

$R^2 = 0.577$
Multiplex real-time quantitative PCR, microscopy and rapid diagnostic immuno-chromatographic tests for the detection of *Plasmodium spp*: performance, limit of detection analysis and quality assurance

Krishna Khairnar³, Donald Martin³, Rachel Lau³, Filip Ralevski³ and Dylan R Pillai*¹,²,³

Discrepancies microscopy-QPCR:
17 positive by QPCR and ICT (treated?)
10 positive by PCR only (……. false positive?)

*Malaria Journal 2009, 8:284*
**Table 6: Comparison of the cost of different techniques employed for the diagnosis of malaria**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Microscopy</th>
<th>BINAXNOW</th>
<th>CARESTART</th>
<th>QPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>QIAmp DNA Extraction</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>$2.30</td>
</tr>
<tr>
<td>TaqMan master mix and probe</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>$3.00</td>
</tr>
<tr>
<td>BINAXNOW</td>
<td>NA</td>
<td>$5.00</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CARESTART</td>
<td>NA</td>
<td>NA</td>
<td>$1.00</td>
<td>NA</td>
</tr>
<tr>
<td>Consumables</td>
<td>$2.50</td>
<td>$0.20</td>
<td>$0.20</td>
<td>$9.50</td>
</tr>
<tr>
<td>Total cost*</td>
<td>$2.50</td>
<td>$5.20</td>
<td>$1.20</td>
<td>$14.80</td>
</tr>
<tr>
<td>Approximate technologist time #</td>
<td>90 mins</td>
<td>30 mins</td>
<td>30 mins</td>
<td>45 mins</td>
</tr>
</tbody>
</table>

* These are approximate cost estimates are in United States Dollars; it does not include tax and shipping

# Technologist time is restricted to hands-on time with sample preparation and testing

hands-on time ≠ time to result
Only limited evaluation in clinical import-malaria settings

Shokoples JCM April 2009: confirmatory test in reference lab: 30 positive patients from Alberta 2007/2008 weekly tests

TABLE 4. *Plasmodium* species identification by microscopy versus PCR during routine testing of patient samples

<table>
<thead>
<tr>
<th>Microscopy</th>
<th>Real-time PCR</th>
<th>Nested PCR&lt;sup&gt;a,b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em> (15)&lt;sup&gt;c&lt;/sup&gt;</td>
<td><em>P. falciparum</em> (15)</td>
<td><em>P. falciparum</em> (15)</td>
</tr>
<tr>
<td><em>P. vivax</em> (5)</td>
<td><em>P. vivax</em> (3), <em>P. ovale</em> (2)</td>
<td><em>P. vivax</em> (3), <em>P. ovale</em> (2)</td>
</tr>
<tr>
<td><em>P. ovale</em> (1)</td>
<td><em>P. falciparum</em> (1)</td>
<td><em>P. falciparum</em> (1)</td>
</tr>
<tr>
<td><em>Plasmodium</em> species other than <em>P. falciparum</em> (4)</td>
<td><em>P. vivax</em> (4), <em>P. ovale</em> (1), <em>P. malariae-</em>&lt;i&gt;P. falciparum&lt;/i&gt; (1)</td>
<td><em>P. vivax</em> (4), <em>P. ovale</em> (1), <em>P. malariae-</em>&lt;i&gt;P. falciparum&lt;/i&gt; (1)</td>
</tr>
<tr>
<td>Suspected mixed infection (1)&lt;sup&gt;d&lt;/sup&gt;</td>
<td><em>P. falciparum</em> (1)</td>
<td><em>P. falciparum</em> (1)</td>
</tr>
<tr>
<td>Unidentified species (2)</td>
<td><em>P. falciparum</em> (2)</td>
<td><em>P. falciparum</em> (2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> PCR results concordant with microscopy results are in bold.

<sup>b</sup> Malaria species identified as *P. falciparum* with suspected *P. vivax* coinfection.

<sup>c</sup> Asterisk indicates level too low for species identification (<0.1%).

<sup>d</sup> Gold standard.

<sup>e</sup> The values in parentheses are the numbers of specimens tested.
conclusions evaluation in clinical import-malaria settings

Shokoples JCM April 2009:
“At the moment, this assay holds great value in support of conventional approaches to malaria diagnosis in correctly identifying malarial species and mixed infections

Khairnar Mal. J. December 2009:
Taken together, QPCR should be implemented in all reference clinical laboratories and used in specific scenarios: (i) when a clinician does not agree with microscopic findings based on clinical pre-test probability of malaria; (ii) when two microscopists disagree on the findings particularly in cases of mixed infection, low parasitaemia, and sample degradation; (iii) when a microscopist does not visualize enough parasite stages to make a speciation call.
Remarkable results from molecular methods: *P. ovale* consists of two major clades, as divergent as different species

http://www.ajtmh.org/cgi/content/full/72/6/719

http://www.cdc.gov/ncidod/EID/vol10no7/03-0411.htm
Remarkable results from molecular methods: *P. ovale* consists of two major clades, as divergent as different species

- Clinical relevance?
Remarkable results from molecular methods: 

*P. ovale* consists of two major clades, as divergent as different species

- Diagnostic relevance
Evaluation of two real time methods: Discrepancies with (mixed infections with) *P. ovale*

<table>
<thead>
<tr>
<th>No. of samples by microscopy&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of samples detected by Rt-PCR with&lt;sup&gt;c&lt;/sup&gt;</th>
<th>No. of samples detected by nested PCR&lt;sup&gt;cd&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2004R, <em>P. ovale</em></td>
<td>2004R, <em>P. malariae</em></td>
</tr>
</tbody>
</table>
More remarkable results from molecular methods

A focus of presumed *P. malariae* in northern Borneo is mostly due to *Plasmodium knowlesi.*
Plasmodium knowlesi
Singh et al Lancet 2004

Figure 3: Detection of Plasmodium and P. knowlesi DNA by nested PCR assays
Molecular size markers in base-pairs (bp) are in the end lanes (M).
our experience

- PCR applied 3 times in past 1.5 year
- 1x disagreement microscopists:
  - Pf or Pf/Pm mixed infection
- 2x disagreement of clinician with microscopy
  - 1x confirmed negative
  - 1x no *Plasmodium* infection, but *Babesia*
Conclusions molecular diagnosis of malaria

– still limited sequence information (specificity)
– time to result is >2 h, cost-effectiveness increases with increasing sample numbers
– published methods not sufficient for primary diagnosis, but useful as confirmatory or secondary test

This afternoon: practical session 5: molecular diagnosis of malaria
Table 3: Discrepant analysis of different assays for the laboratory diagnosis of malaria based on results of QPCR.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pf</em></td>
<td>85</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Pv</em></td>
<td>54</td>
<td>5</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Pm</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Po</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Pf &amp; Pv</em></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Pm &amp; Po</em></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Pf &amp; Pm</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>266</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>145</td>
<td>276</td>
<td>2</td>
<td>5</td>
<td>18</td>
<td>18</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Real time PCR

• advantages: shorter turn around time
• additional identification of PCR product through hybridization or melting curve