



ARMY MEDICINE
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Role of Malaria Detection Methods for Enrollment and Outcomes in Clinical Trials

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Objectives

- Overview of malaria detection methods
- Considerations for enrollment
- Measuring Outcomes
 - PCR ‘Correction’ of Microscopy
 - Measures of parasite clearance
 - PCR ‘Adjustment’ of Treatment Outcomes
 - Considering possible outcomes
 - Differentiating new infection from recrudescence
 - Considerations for *P. vivax*

I. Malaria Detection in Clinical Trials

- Rapid diagnostic tests should be limited to screening potential subjects in regulated trials.
- Microscopy remains the most widely used method, and offers the advantage of being able to visualize parasite stages. However, it is less sensitive than PCR, and can have a high false positive rate.
- PCR is the most sensitive and specific method, but on-site performance in real time remains limited. PCR can now quantitate parasitemia comparable to microscopy.
- Efficacy results of trials should be 'PCR-adjusted' to determine final outcomes, but results should be interpreted carefully. Reinfection ranges from <10% in low transmission settings to >50% in holoendemic areas.

Rapid Diagnostic Tests

- Large variety of RDTs now available including FDA-approved tests (eg Binaxnow)
- Most are lateral flow immunoassays
- Sensitivity of some tests approaches that of microscopy for though specificity not as good
- Not useful for follow-up
- Do not provide a permanent specimen result – cannot be reread or re-run
- Can lead to treatment of false positives
- Generally unsuitable for clinical trial enrollment purposes, though useful screening tool for recruiting

Microscopy

- Remains the “gold standard”
- Most widely available method
- Generates real time, actionable results
- Can identify species and parasite stages
- Results within 0.5-2 hours
- Relatively inexpensive, low tech method compared to PCR

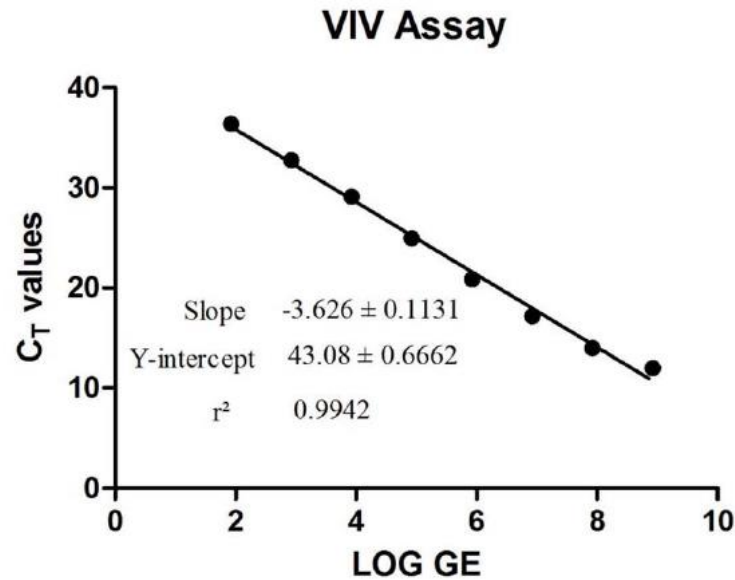
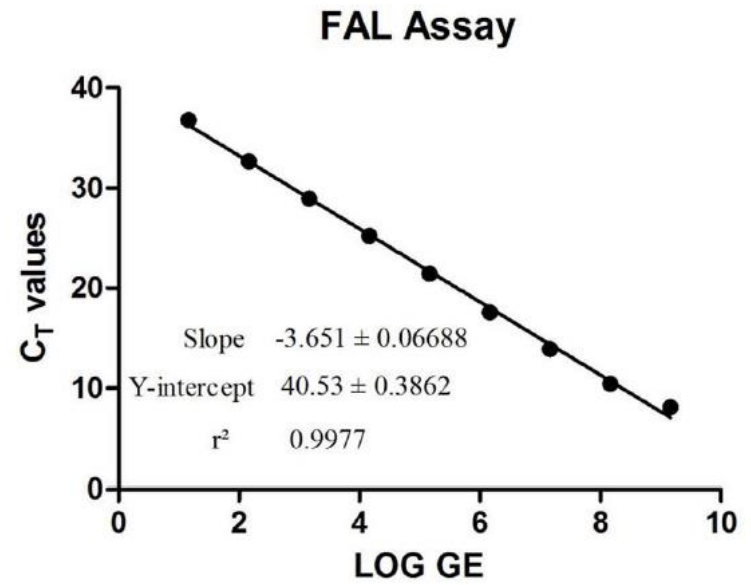
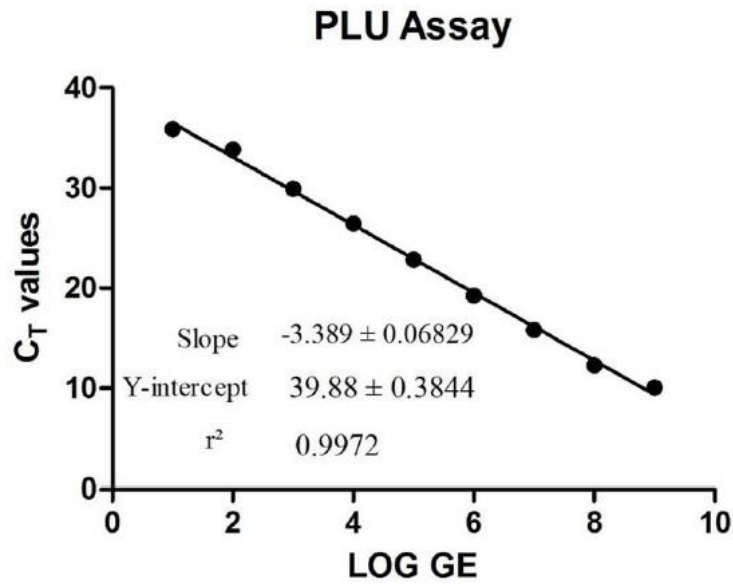
Microscopy Considerations

- Requires considerable training – 1-3 years to adequately train an expert microscopist
- Sensitive to ~10 parasites/microliter
- WHO competency exam can estimate individual reader sensitivities/specificities
- 3 reader paradigm recommended – ‘A’ and ‘B’ do independent blinded initial reads
- Expert level ‘C’ reader does blind over-read of non-concordant results

Molecular Methods

- RT-PCR testing increasingly available on-site to generate same-day results
- More sensitive than microscopy (~ 2 -log)
- Highly specific - can be multiplexed to detect multiple species in single run
- Special methods for Gametocyte detection
- Requires significant infrastructure, training and quality control measures; expensive

qPCR



Kamau E, Alemayehu S, Feghali KC, Saunders D, Ockenhouse CF (2013) Multiplex qPCR for Detection and Absolute Quantification of Malaria. *PLoS ONE* 8(8): e71539.

Figure 2. Linear regression plots for absolute qPCR assays. Real-time PCR assays were performed using plasmid DNAs for each assay. Plasmid DNA was 10-fold serially diluted at each point and ran in 4–8 replicates. A linear regression plot was generated using GraphPad Prism. The slope, the Y-intercept and the r^2 value were determined. Data shown confirms that these assays perform with high efficiencies.

doi:10.1371/journal.pone.0071539.q002

Quantitative Microscopy vs. qPCR

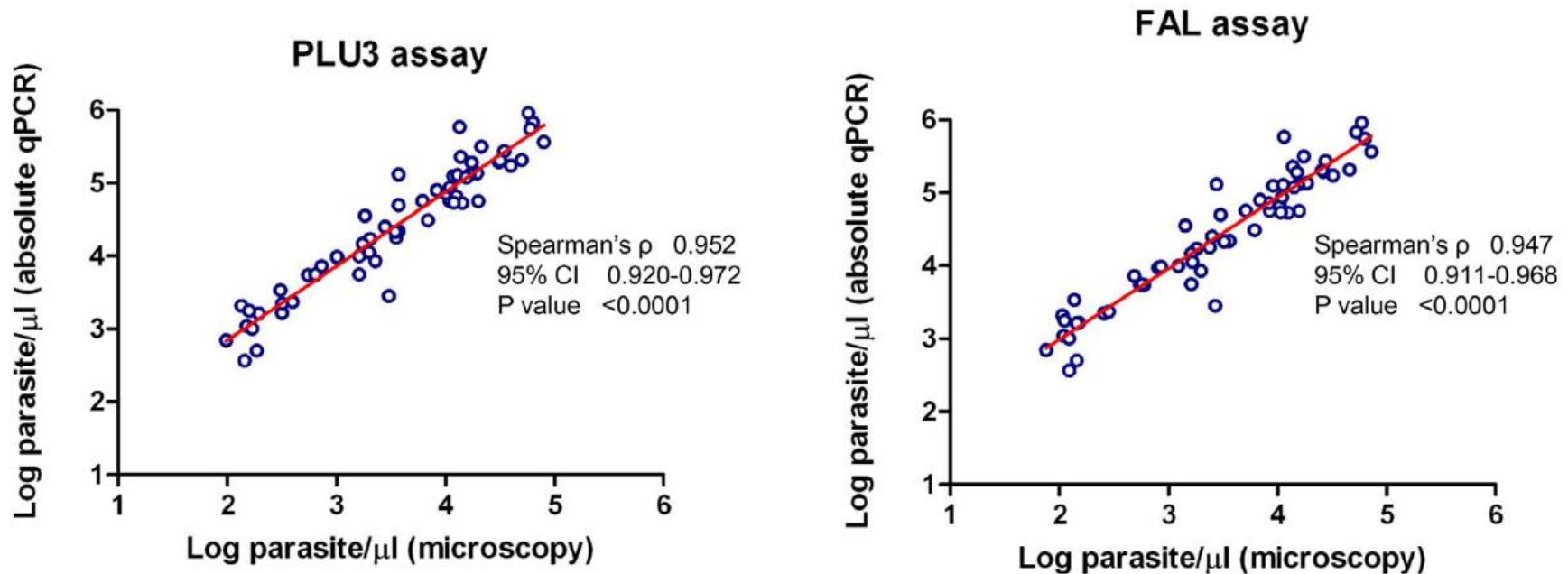


Figure 3. Analysis of parasite densities in clinical samples using absolute qPCR and microscopy. Absolute quantitative qPCR was performed using plasmid DNA as the standard to analyze clinical samples. The \log_{10} parasite densities in terms of parasite/ μl was determined from qPCR assays and compared to the \log_{10} parasite densities as determined by expert microscopist. The correlation coefficient of parasite densities measured using the two methods was calculated using the nonparametric Spearman correlation coefficient. There was a statistically significant correlation between parasite density measured by microscopy and absolute quantitative qPCR.

doi:10.1371/journal.pone.0071539.g003

Considerations for Enrollment

- Microscopy remains the gold standard for enrolling subjects in clinical trials
- Microscopy rarely misses clinically apparent infections, but **may miss subclinical infections**
- RT-PCR methods highly sensitive and specific but **rarely available in real time**
- **RDTs should be used for initial screening only, should be confirmed by another method**

II. Measuring Outcomes

Detection methods play important roles in assessing trial outcomes

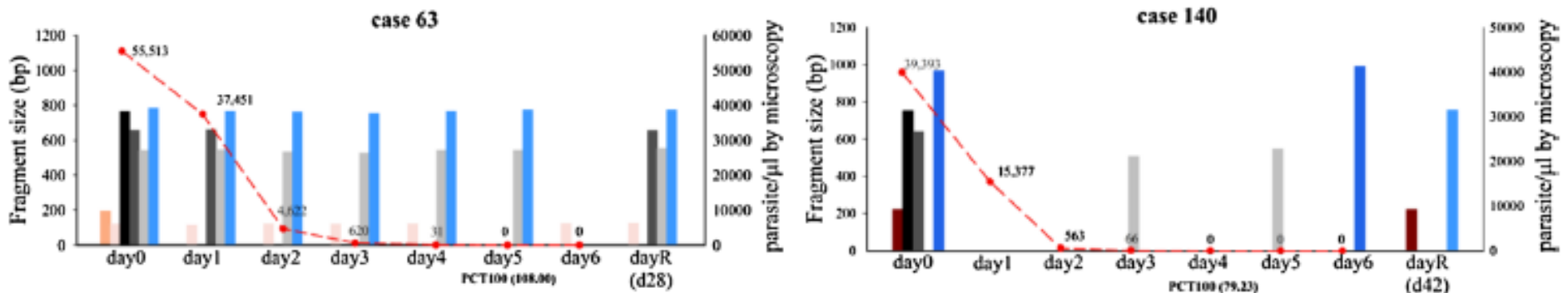
1. Parasite clearance – PCR often remains positive after apparent microscopic clearance
2. Assessing recurrences – RT-PCR can be used to ‘correct’ the microscopy result
3. Distinguishing true recrudescence/treatment failure from reinfection, relapse, *P. vivax*, etc

PCR 'Correction' of Microscopy

- Critical for accurate outcome measures
- Recurrences are often detected subclinically
- Low parasitemia makes microscopic detection and distinction of *P.f.*, *P.v.*, etc challenging
- Routine PCR correction may detect submicroscopic infection during follow-up
- Major Challenge – rarely available in real time
- Clinical significance may be unclear
- Recommended as part of post-hoc analysis

Measures of Parasite Clearance

- Parasite clearance measurements are important for some trial outcomes – e.g. resistance studies
- Parasite density often calculated based on blood cell counts using standard formulas – **can be inaccurate**
- PCR is ~2 log more sensitive than microscopy – has **substantial implications for ‘clearance’**



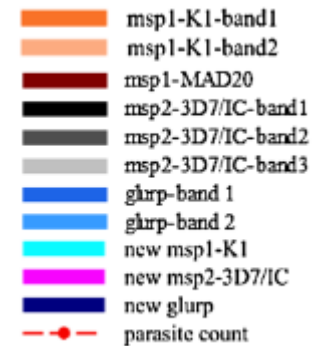
PCR 'Adjustment' of Trial Outcomes

There are many possible treatment outcomes

- *P.f.* → no return for specified period = ACPR
- *P.f.* → same *P.f.* = recrudescence
- *P.f.* → different *P.f.* = reinfection
- *P.f.* → *P. vivax* = ACPR??; **ROUGHLY 1/3 in SE Asia will have blood stage *P.v.* following *P.f.* treatment**
- Mixed *P.f./P.v.* → *P.f.* = *reinfect or recrudescence?*
- *P.f./P.v.* → *P.v.* = relapse, reinfection, recrudescence

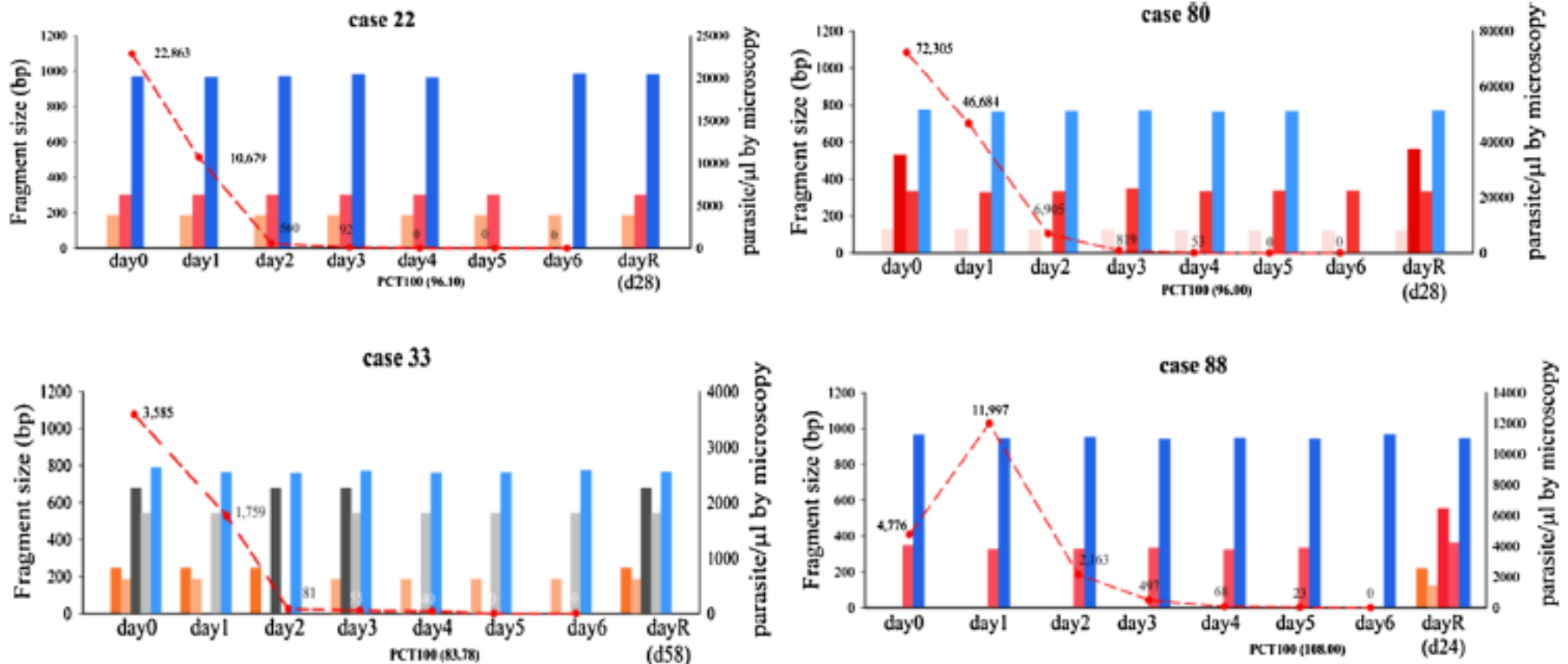
Use of Parasite Genotyping

1. Current standard is genotyping parasite MSP-1, MSP-2 and Glurp.
2. Variants can be used to distinguish reinfection from recrudescence.
3. Genotyping often reveals polyclonal infections.
4. Reinfection rates vary widely from >10% in SE Asia to >50% in Africa.

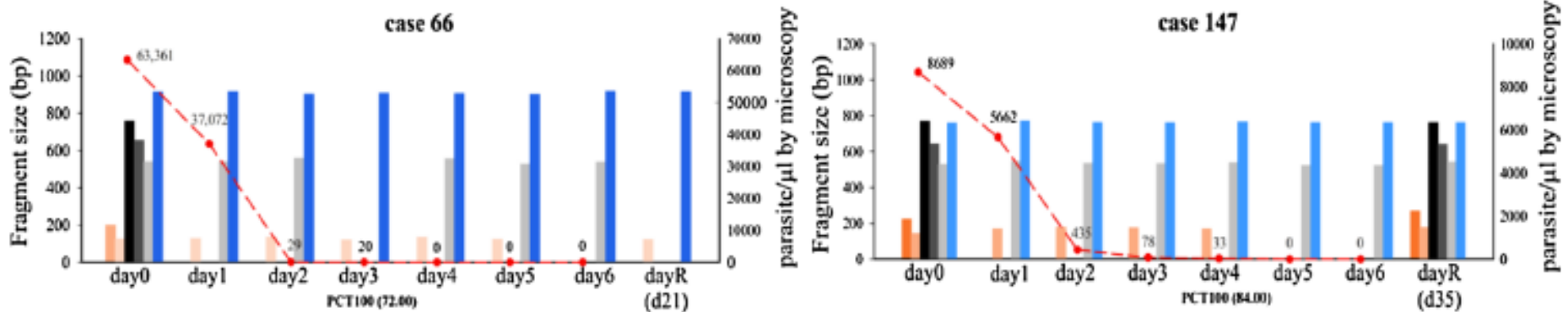


A. Recrudescence cases (n=8)

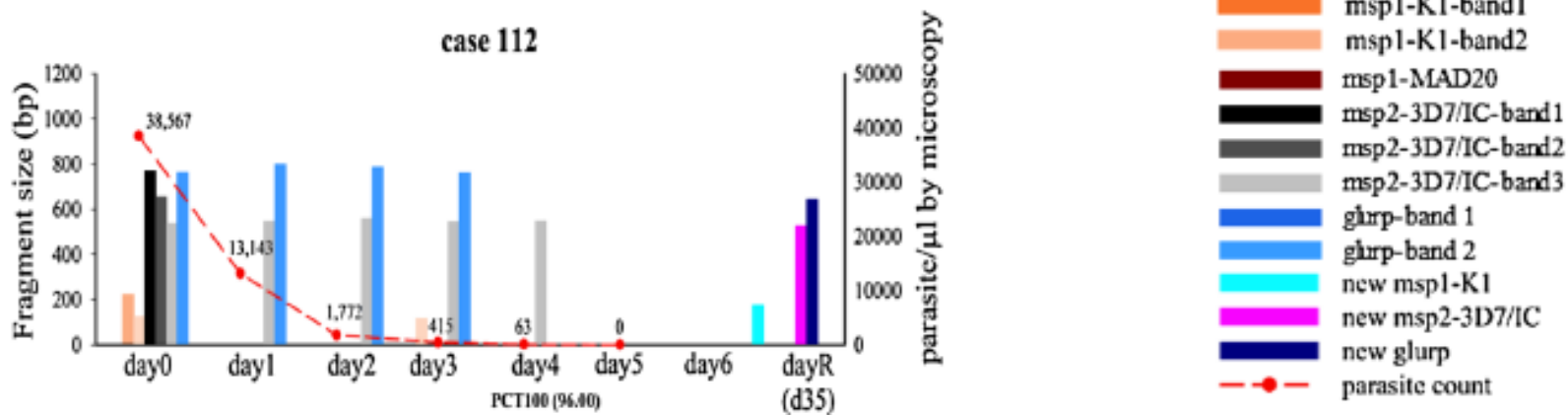
Gosi et al. Malaria Journal 2013, 12:403



Molecular Fingerprinting – P.f.



B. New infection (n=1)



Considerations for *P. vivax* – multiplicity of infection

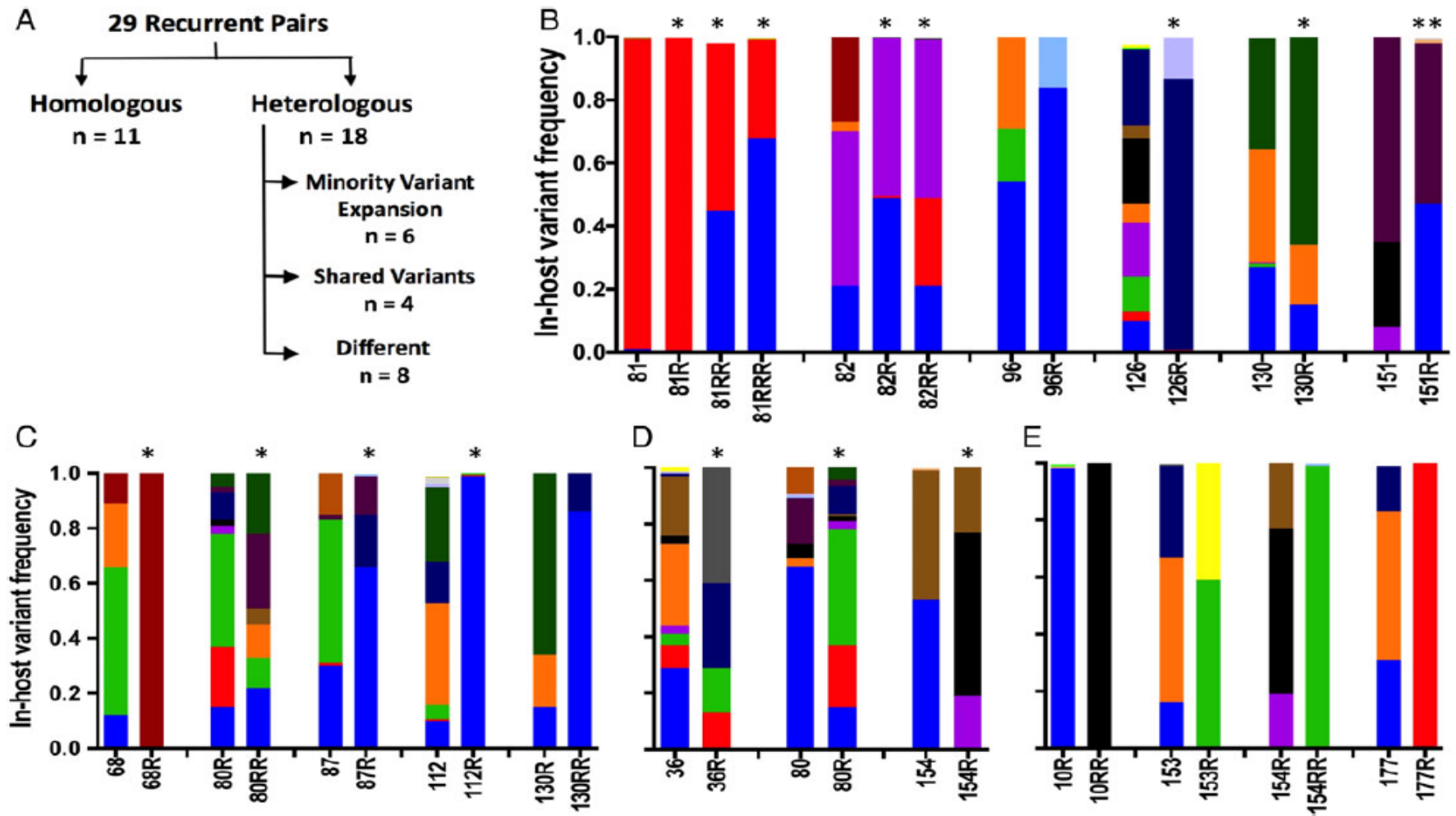


Figure 3. Representative genotypes of recurrent pairs categorized into homologous or heterologous pairs (A) based on overlap of *pvmsp1* variants in the recurrent and preceding infection. Unique haplotypes are represented by specific colors across all samples. B, Homologous pairs were defined as having the same dominant or codominant haplotype at recurrence as seen in the preceding episode. C, In pairs exhibiting minority variant expansion, a minority parasite population existing at <20% in-host frequency in the initial infection reappeared as the dominant variant at recurrence. D, At least 1 shared variant defined our third category, while one-third of heterologous pairs shared no overlap at all (E). Pairs identified as probable relapses based on statistical testing are denoted by an asterisk. A pair that was “indeterminate” by statistical testing but judged as likely relapse based on microsatellite results are denoted by double asterisks. The genotypes of all 29 pairs are depicted in [Supplementary Figure 3](#) (21 depicted here).

P. vivax – it's complicated

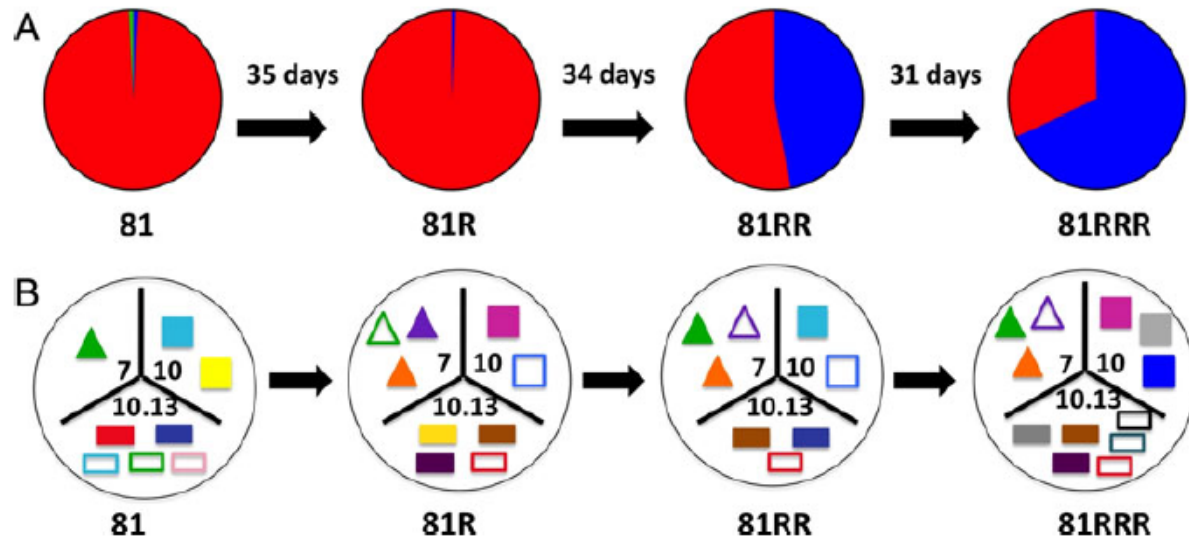


Figure 4. Pictorial representation of *pvmsp1* haplotypes and microsatellite alleles MS7, MS10, and MS10.13 found in patient 81 through 4 consecutive *P. vivax* parasitemic episodes. In Panel A, pie slices reflect the proportion of sequencing reads assigned to each *pvmsp1* haplotype variant within each episode (CAM.00 in red, CAM.01 in blue, CAM.51 in yellow). In Panel B, microsatellite alleles for PvMS7, PvMS10, and MS10.13 are depicted as different colored triangles, squares, and rectangles, respectively, within each tripart segment. Alleles that appeared only as minor peaks (less than one-third height of the dominant allele peak) are depicted as hollow shapes.

Summary

- Rapid diagnostic tests should be limited to screening potential subjects in regulated trials.
- Microscopy remains the most widely used method, and offers the advantage of being able to visualize parasite stages. However, it is less sensitive than PCR, and can have a high false positive rate.
- PCR is the most sensitive and specific method, but on-site performance in real time remains limited. PCR can now quantitate parasitemia comparable to microscopy.
- Efficacy results of trials should be 'PCR-adjusted' to determine final outcomes, but results should be interpreted carefully. Reinfection rates vary widely based on endemicity ranging from <10% in SE Asia to >50% in Africa. *P. vivax* evaluation is complex.
- Standardization of methodology and outcome is critically important.

Thank you!

