



Application of Real-Time Quantitative PCR (qPCR) in Anti-malarial Clinical Trial

Davis Nwakanma, Eniyou Oriero, Sanie Sesay, Lesong Conteh, David Conway

MALARIA DIAGNOSIS

- **CLINICAL DIAGNOSIS:**
 - BASED ON SYMPTOMS (Inaccurate due to lack of Specificity)

- **LABORATORY DIAGNOSIS:**
 - SLIDE MICROSCOPY
 - ANTIGEN TESTS (RDTs)
 - PCR ASSAYS

Rationale for Molecular Diagnosis of Malaria

- To circumvent difficulties associated with use of slide microscopy in clinical trials:
 - Delay in completion of studies due to slow pace of microscopy
 - Low sensitivity of detection of low-grade infections
 - Operator induced variability
 - Labour intensive
 - Higher sensitivity would allow **more precise determination of clinical trial end points** such as parasite clearance time (PCT) and time-to-blood infection.

OBJECTIVES

- Establish a qPCR method for routine application in determination of malaria parasitaemia.
- Optimize this method in a clinical trial.
- Evaluate agreement of parasite density estimates obtained from qPCR with blood film microscopy
- Compare unit cost of qPCR method with slide microscopy for malaria diagnosis.

Real-Time Quantitative PCR

- **Assay Development:**
- Evaluated different published protocols & primers for quantitative amplification of malaria parasite DNA (RANK, PLASMO, MACH).
- Evaluated SYBR Green versus TaqMan probe-based qPCR.
- Selected the most sensitive primer /detection method combination.
- The combination of **PLASMO primer with TaqMan** probe assay had the most sensitive and robust quantitation.
- Optimize assay for high throughput sample processing (96-well plate format, each sample in duplicate, use of multichannel pipetting & master mix trough).

Evaluation of qPCR method

- **OPD STUDY:**

- Recruited 1211 suspected malaria cases at the outpatient clinic.
- All age groups included.
- Obtained ~250 μ L of fingerprick blood sample for DNA extraction and thick film microscopy.

- **PYRAMAX:**

- Samples from efficacy trial of Pyramax® (pyronaridine artesunate, 3:1) vs Coartem® (artemether lumefantrine).
- 1060 Blood Samples from 106 patients, each sampled at 10 time points (every 8h) over a 3-day period.
- Approximately 250 μ L of blood was collected for DNA extraction and thick film microscopy.

Methods

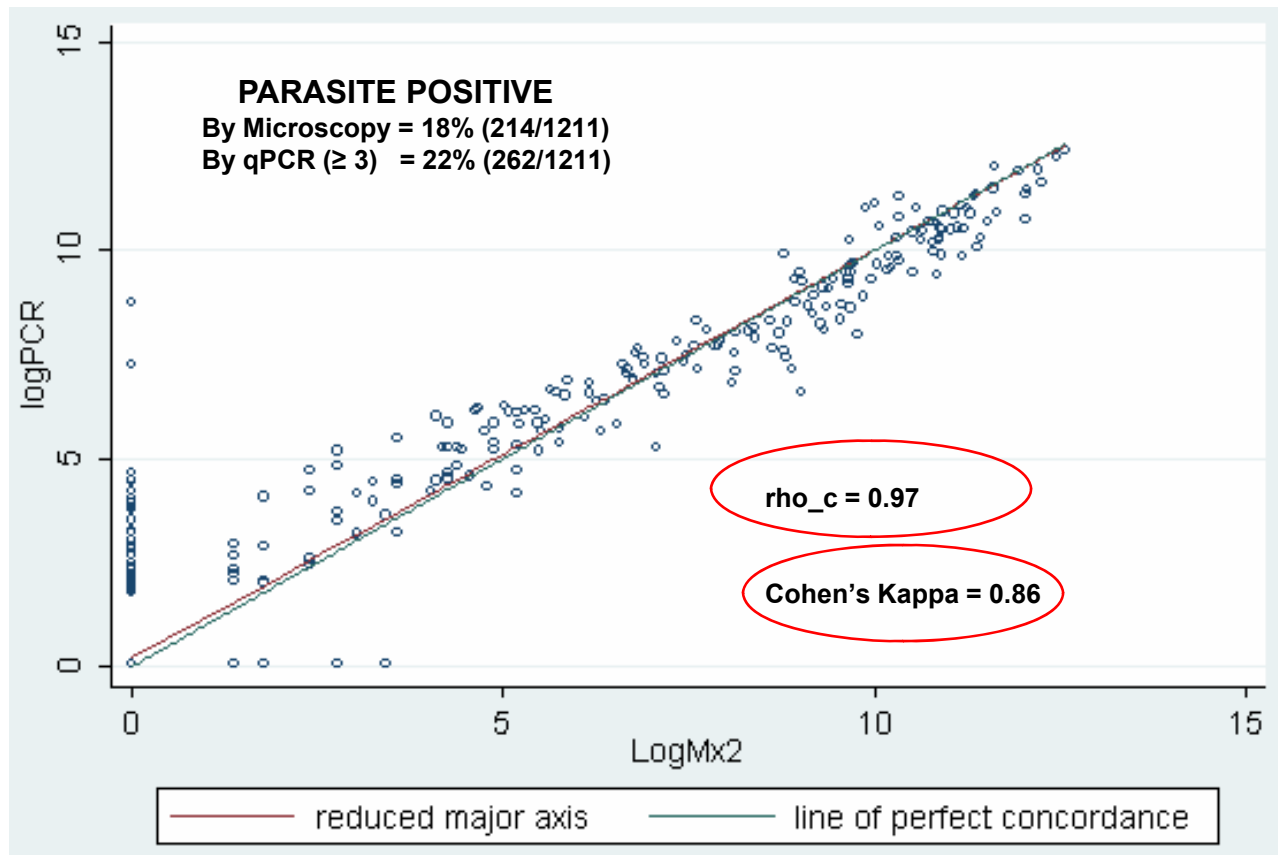
- **Microscopy:**
- Two independent microscopic examinations of 200 HPF of Giemsa-stained blood films for detection.
- Parasite density estimated by counting 100 HPF (0.2 μ l blood) using the GW method.
- Discordant reads resolved by a 3rd Microscopist.
- **PCR-based Methods:**
- TaqMan qPCR assay using PLASMO primer based on amplification of *Plasmodium* 18s rDNA.
- Parasite density estimation by qPCR analysis of 7.5 μ l blood per assay in duplicate.
- And quantitation using DNA standards generated from serial dilutions of 3D7 parasite culture.

Methods

- **Statistical Analysis:**
- Agreement between parasite detection by microscopy and qPCR was measured using Kappa Coefficient (Cohen's Kappa).
- Parasite count data were log transformed.
- Scatter plots to visualize the linear correlation of parasite density estimates by Microscopy and qPCR.
- Agreement of parasite density estimates determined by Lin's concordance correlation coefficient and assessed graphically using Bland-Altman plots.
- Survival analysis to compare parasite-clearance-time (PCT) determined by microscopy and by qPCR at PC 99.

RESULTS (OPD Study)

Agreement of Microscopy with qPCR estimates of Malaria Parasite Density

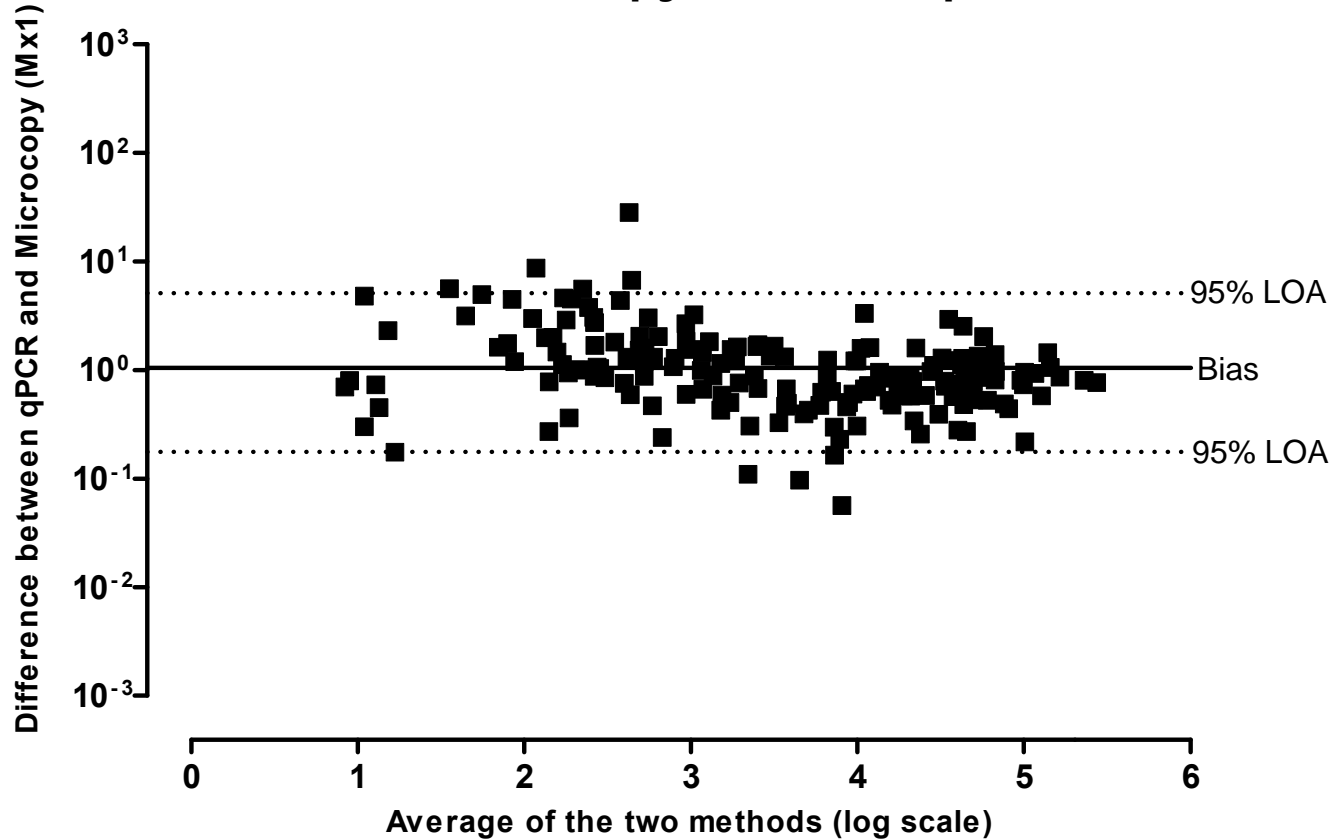


Concordance of microscopy parasite count with qPCR estimates of parasite density in an outpatient population of suspected malaria cases.

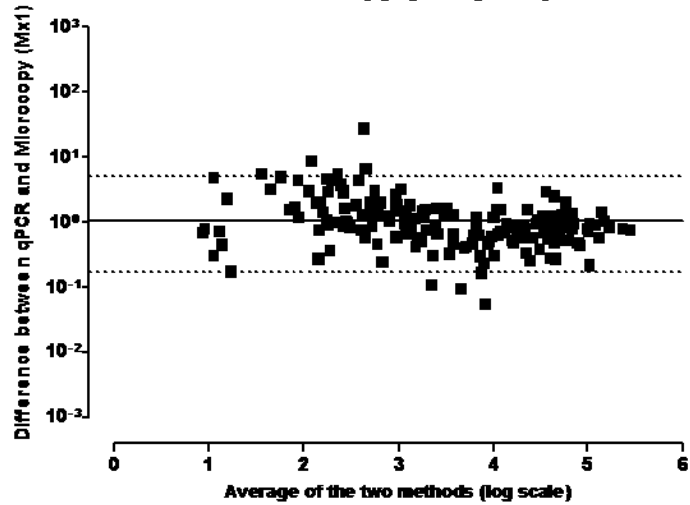
(Lin's concordance correlation coefficient = $\rho_c = 0.97$; 95% CI = [0.964-0.972])

Comparison of Methods

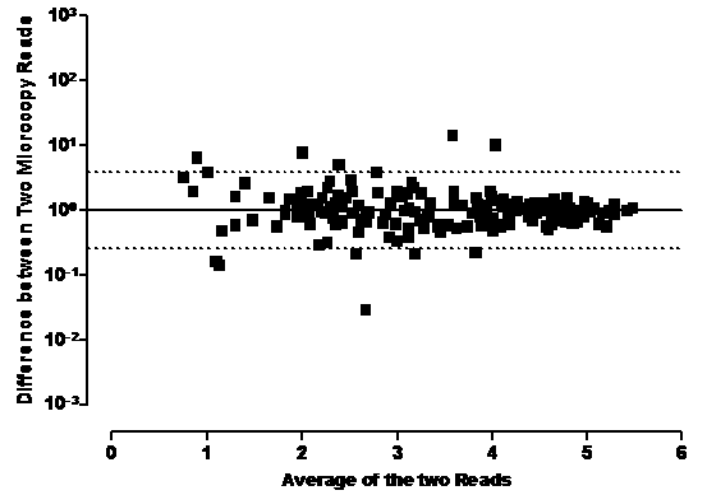
Bland-Altman Plot of Microscopy (Mx1) vs qPCR Parasite Density



Bland-Altman Plot of Microscopy (Mx1) vs qPCR Parasite Density

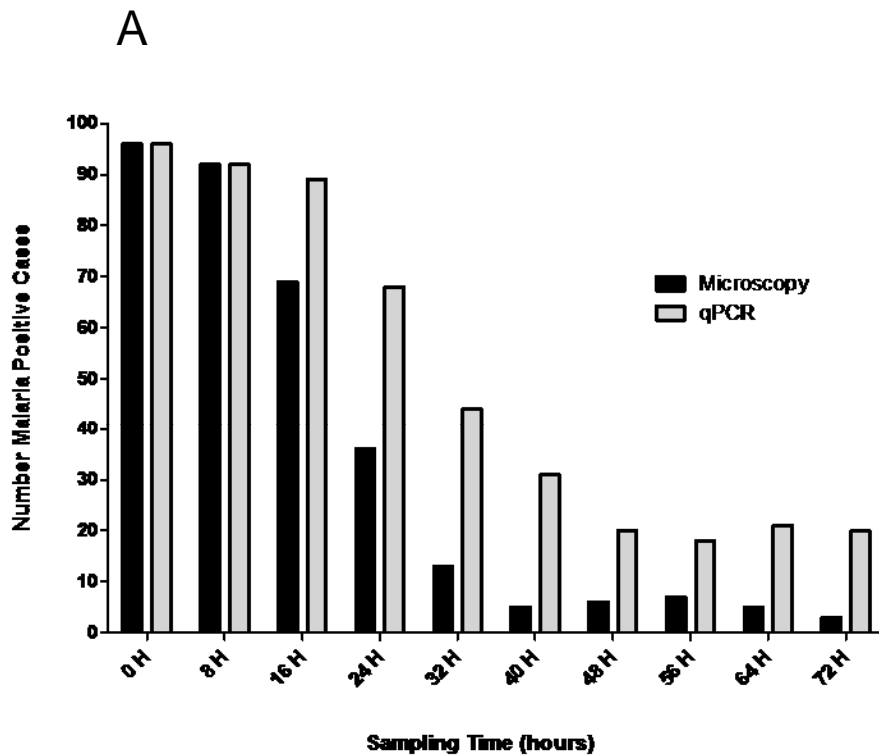


Bland-Altman Plot of 1st and 2nd Microscopy of the same slide



RESULTS (Pyramax Study)

Parasite Clearance Time Microscopy vs qPCR



Parasite Clearance Time Microscopy vs qPCR
(No Gametocytes)

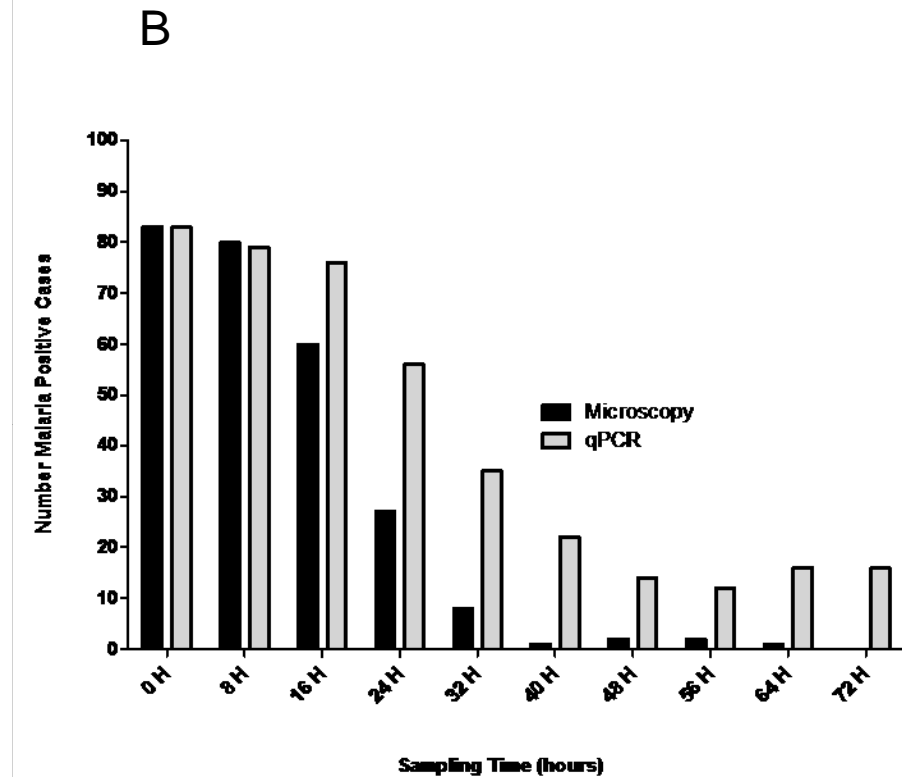
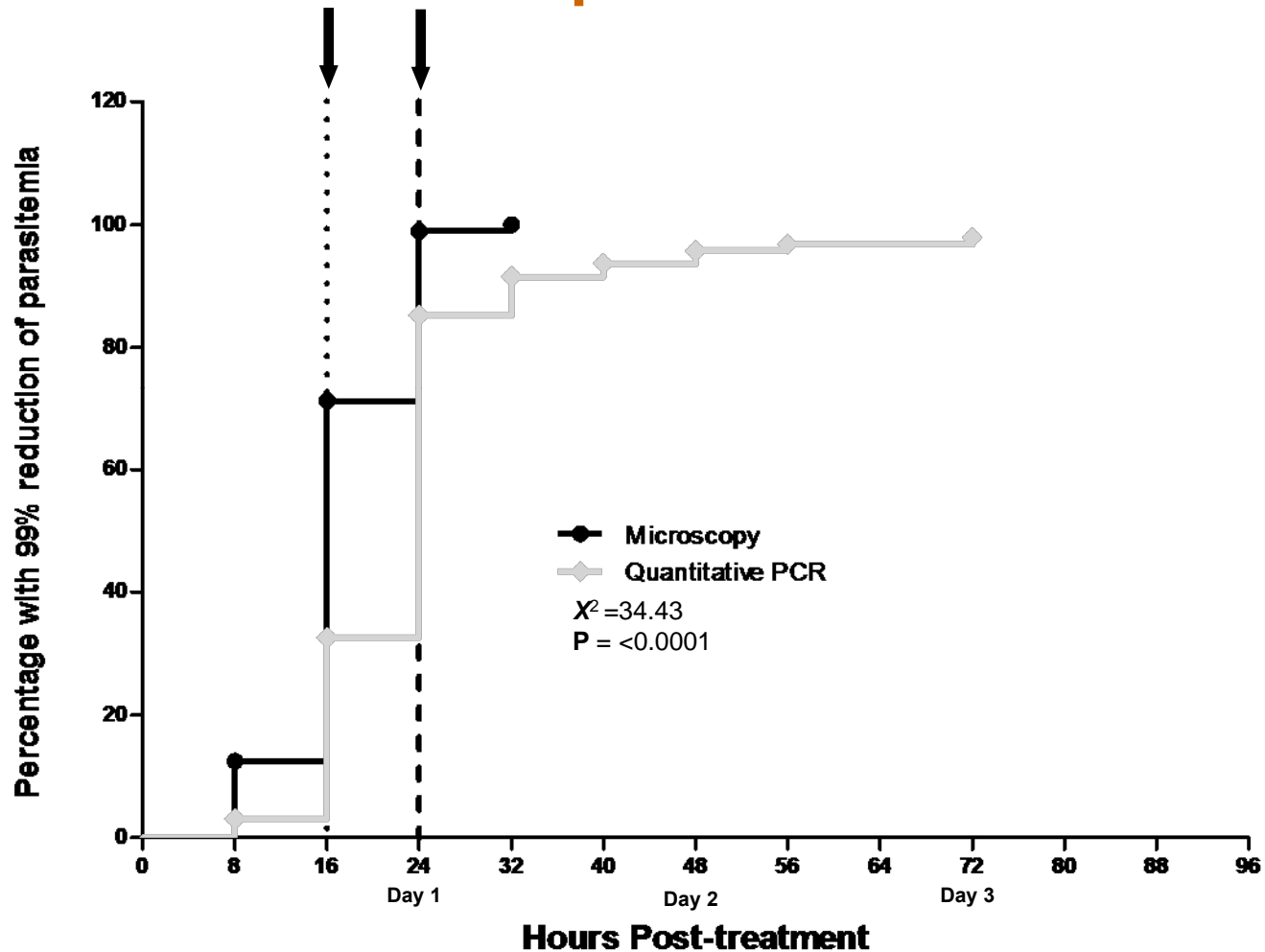


Figure: Parasite clearance in drug-treated patients monitored by qPCR and Microscopy over a 3-day period following treatment in all patients (A) and in patients with asexual parasites only (B). At 72h post-treatment it was possible to detect parasites by qPCR in ~20% of patients in whom Microscopy did not detect any infection

Parasite Clearance Curve by Mx & qPCR

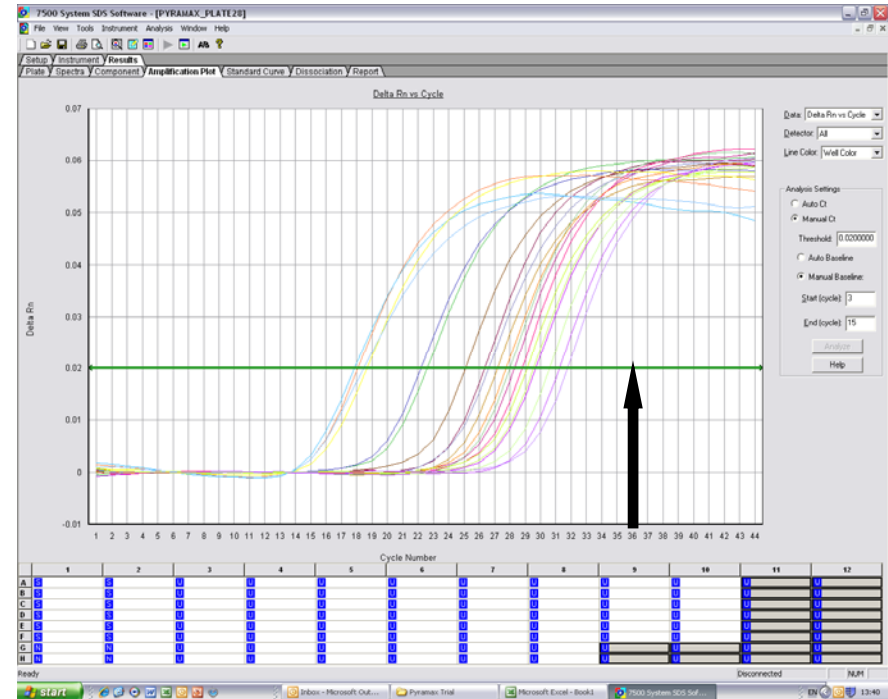
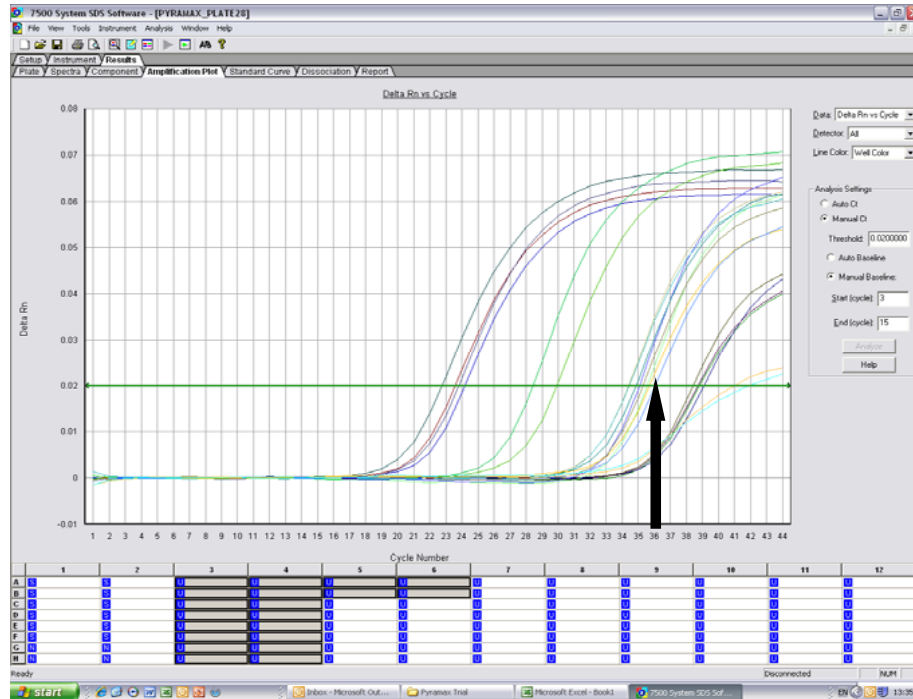


Time to clearance of 99% of initial parasitemia estimated by Microscopy and qPCR

Drug Failure (DF) Vs Adequate Parasitological Cure (APC) By Microscopy & qPCR

‘Adequate Parasitological Cure’: SSN 2698

‘Drug Failure’: SSN 3596



SSN	0hr	8hr	16hr	24hr	32hr	40hr	48hr	56hr	64hr	72hr
2698-qPCR	2545	3360	103	2	2	4	1	0	0	0
2698-Mx	34600	29255	38900	85	0	0	0	0	0	0

SSN	0hr	8hr	16hr	24hr	32hr	40hr	48hr	56hr	64hr	72hr
3596-qPCR	144000	113223	6651	665	141	151	92	47	20	16
3596-Mx	256850	164500	6990	60	20	0	5	0	0	0

Assay Throughput & Cost Comparison

Based on 1000 samples	Microscopy	qPCR
Time: 8hrs/day; 240 days/year		
Lab Tech (100%)	2	2
Supervisor (10%)	1	1
Samples/day	25	176 (2x88)
Project Time	40 days	8 days
Personnel Cost	£ 1180 (67%)	£ 236 (5%)
Cost of Supplies	£ 442 (23%)	£ 4088 (84%)
Capital Cost (usage, discounted)	£149 (8%)	£ 537 (11%)
Full Economic Cost per sample	£ 1.77	£ 4.86

- qPCR is 5X faster than Slide Microscopy but also 2.5X more expensive

Conclusions

- qPCR has higher sensitivity than microscopy but estimate of parasite density obtained is comparable to microscopy parasite count.
- Application of qPCR method could lead to more precise determination of relative efficacies of anti-malarial drugs.
- The higher throughput of qPCR assay should facilitate malaria surveillance especially in the current C/E era.
- qPCR is 2.5X more expensive but increases sample processing time 5X over microscopy.
- The detection limit of Slide Microscopy **does not represent a biologically meaningful threshold.**
- Routine use of more sensitive detections method could lead to **earlier detection of increasing drug tolerance** before clinical manifestation as resistance.



Acknowledgements



E D C T P



Eniyou Oriero, Lamin Touray, Lamin Kuyateh, Ndey Fatou Drammeh
Our Study Participants
Malaria Programme Staff, Medical Research Council Laboratories, The Gambia
FUNDING: European & Developing Countries Clinical Trials Partnership (EDCTP)



Thank you

