Molecular diagnostics of malaria;

is full automatisation possible considering the current guideline?

Foekje F. Stelma
Foekje.Stelma@Radboudumc.nl

Radboudumc

Decreasing number of imported cases with malaria

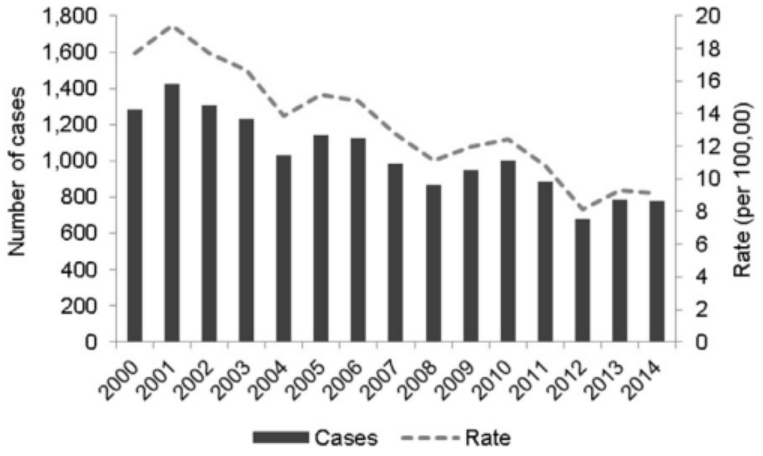
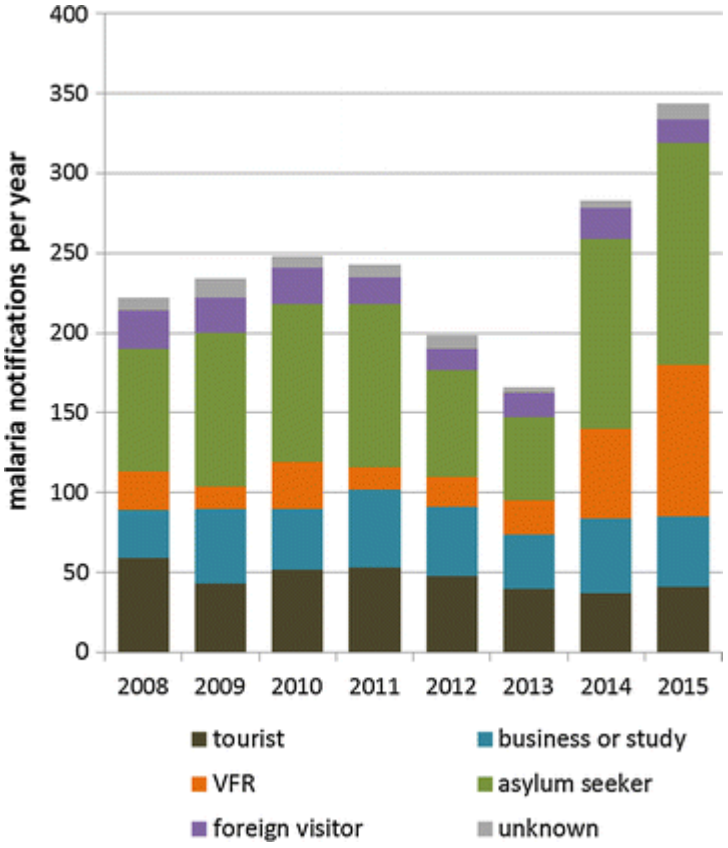


Fig. 1. Total number of cases and rates per 100,000 population of malaria by year in London, 2000–2014.



E. Rees et al. / Travel Medicine and Infectious Disease 17 (2017)
*De Gier et al.. Malaria Journal2017**16**:60*

Keeping up expertise is becoming a problem

- 250-350 positive cases in the Netherlands per year (de Gier 2017)

- Radboudumc; 142 diagnostic requests; 91 patients in 2017
- 14% positive
  - *P. falciparum* 5
  - *P. ovale* 2
  - *P. vivax* 2
  - *P. malaria* 1
  - Double infection (p.f, p.o) 1

- 8 qualified technicians on call 24/7

- All receive regular training

NOT cost effective

Many labs turn to RDT's, but…

- Non-sensitivity for all plasmodium species Inability to detect low level infections (less than 200 parasites per µl)
- False positives

v. Gool ECCMID 2017

147 patients with active malaria (asexual stages present in bloodsamples)*

Positive patients according to Gold Standard of Positivity*			Results methods under investigation							
			illumigene ® Malaria	*illumigene* ® Malaria PLUS	Binax Now ICT®					
No. of patients	Malaria (sub)species	Parasitaemia parasites/ul range	no. patients with correct diagnosis of malaria (sensitivity %)	no. patients with correct diagnosis of malaria (sensitivity %)	No. positive tests, irrespective of type of band (sensitivity %)	No. tests with correct band(s) for species present (sensitivity %)	Reactive bands: HRPII and/ or aldolase			
							Negative	HRPII only	HRPII and aldolase	Aldolase only
102	*P. falciparum*	27-990.000	102 (100)	102 (100)	102 (100)	101 (99)	0	33	68	1**
28	*P. vivax*	132-73.650	28 (100)	28 (100)	21 (75)	21 (75)	7	0	0	21
7	*P. ovale* (W 5 +C 2)	564-32.200	7 (100)	7 (100)	2 (29)	2 (29)	5	0	0	2
4	*P. malariae*	40-90.000	4 (100)	4 (100)	1 (25)	1 (25)	3	0	0	1
1	*P. knowlesi*	270.000	1 (100)	1 (100)	1 (100)	1 (100)	0	0	0	1
3	MI: *P. falciparum* and *P. malariae*	1890-4087	3 (100)	3 (100)	3 (100)	1 (33)	0	0	1	2
2	MI: *P. falciparum* and *P. ovale*	0,2-0,3	2 (100)	2 (100)	2 (100)	0 (0)	0	2	0	0
Total: 147			147 (100 %)	147 (100 %)	132 (90 %)	127 (86 %)	15	35	69	28

Legend:
* Only first sample before treatment (no follow up samples after start of treatment) used.
** Sample of patient with proven HRPII gene deletion in *P. falciparum* isolate.
† *P. knowlesi* morphologically stongly resembles *P. malariae:* defintive determination only possible with PCR.
W= *P. ovale wallikeri* (no. 5), C = *P. ovale curtisi* (no. 2)

red = incorrect result reactive band

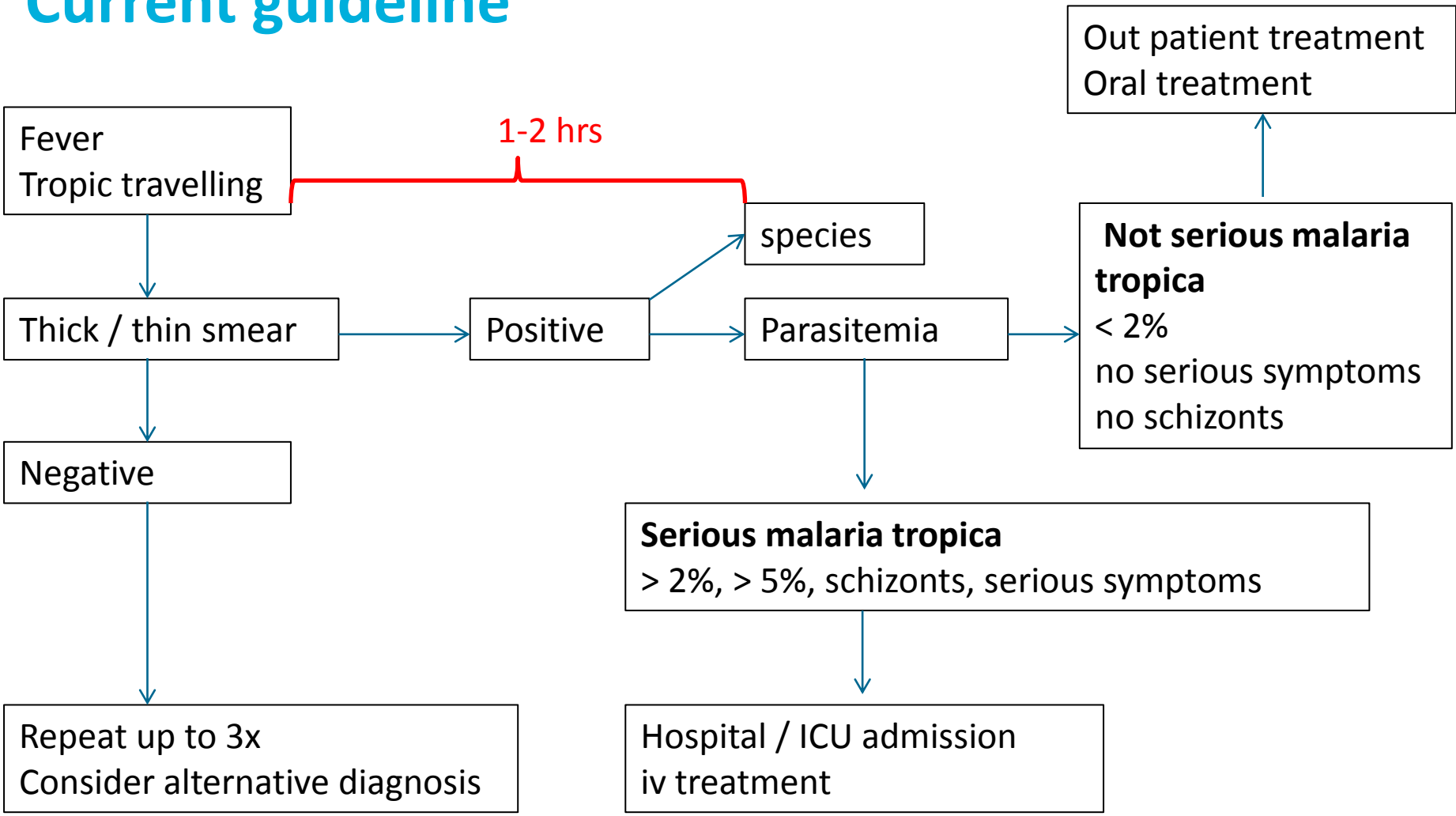
Solutions DNA based diagnostic techniques ?

- PCR LoD as low as 0.5 – 5 parasites/ml

BUT

- Well trained technicians and expensive reagents → high costs
- High profile and equipped laboratories
- Prone to contamination and amplification of non-targeted DNA sequences

Current guideline



Does molecular diagnostics fit in?

Technical issues

- Clinical and analytical sensitivity
- Species determination (malaria tropica versus malaria tertiana / quartana)
- Stage determination (trophozoites, schizonts, gametocytes)
- Quantification

Logistical issues

- 24/7
- Standardization and harmonisation of techniques within a laboratory
- Run time
- First diagnosis versus follow up

Clinical versus analystical sensitivity

Method	Parasites / ml	Parasitaemia
QBC[1,2]	60.000	0,001%
Thick smear[1,2]	20.000	0,001%
RDT[5]	50.000	0,001%
LAMP[3,4]	2000	0,0002%
qPCR[3,4]	2-50	0,000002%

Much depends upon input volume[4]

1. *Baird et al. Diagnosis of malaria in the field by fluorescence microscopy of QBC capillary tubes. Trans R Soc Trop Med Hyg. 1992 Jan-Feb;86(1):3-5*
2. *G.O. Adeoye, I.C. Nga. Comparison of Quantitative Buffy Coat technique (QBC) with Giemsa-stained thick film (GTF) for diagnosis of malaria. Parasitology International 56 (2007) 308–312*
3. *Britton et al. Novel molecular diagnostic tools for malaria elimination: a review of options from the point of view of high-throughput and applicability in resource limited settings. Malar J (2016) 15:88*
4. *Imwong M, Hanchana S, Malleret B, et al. High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias. J Clin Microbiol 2014;52:3303–9.*
5. *Adu-Gyasi et al. Assessing the performance of only HRP2 and HRP2 with pLDH based rapid diagnostic tests for the diagnosis of **malaria** in middle Ghana, Africa.PLoS One. 2018 Sep 7;13(9)*

Detection limits of molecular tests

- Volume of blood analysed [1]
- Copy number of the amplified molecular marker serving as the template for amplification[2]
  - Single- or low-copy 18S rRNA genes
  - Mitochondrial DNA higher number of copies

1. *Imwong M, Hanchana S, Malleret B, et al. High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias. J Clin Microbiol 2014;52:3303–9.*
2. *Gruenberg et al. Plasmodium vivax molecular diagnostics in community surveys: pitfalls and solutionsMalar J (2018) 17:55*

qPCR versus microscopy

18S rRNA gene

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
** Alam 2011	20	6	1	311	0.95 [0.76, 1.00]	0.98 [0.96, 0.99]
** Alam 2011	166	4	5	163	0.97 [0.93, 0.99]	0.98 [0.94, 0.99]
Boonma 2007	86	5	3	42	0.97 [0.90, 0.99]	0.89 [0.77, 0.96]
Chaudry 2012	30	3	0	27	1.00 [0.88, 1.00]	0.90 [0.73, 0.98]
Cheng 2013	16	3	0	133	1.00 [0.79, 1.00]	0.98 [0.94, 1.00]
Chou 2012	389	33	0	578	1.00 [0.99, 1.00]	0.95 [0.92, 0.96]
Dawoud 2008a	60	6	0	24	1.00 [0.94, 1.00]	0.80 [0.61, 0.92]
Elahi 2013	207	1	0	119	1.00 [0.98, 1.00]	0.99 [0.95, 1.00]
Genc 2010	44	12	0	36	1.00 [0.92, 1.00]	0.75 [0.60, 0.86]
* Hwang 2011	80	32	0	0	1.00 [0.95, 1.00]	0.00 [0.00, 0.11]
Khan 2013	110	13	0	177	1.00 [0.97, 1.00]	0.93 [0.89, 0.96]
Nkrumah 2010	178	110	4	197	0.98 [0.94, 0.99]	0.64 [0.59, 0.70]
Rakotonirina 2008	104	4	0	205	1.00 [0.97, 1.00]	0.98 [0.95, 0.99]
Ratsimbasoa 2012	197	74	0	258	1.00 [0.98, 1.00]	0.78 [0.73, 0.82]
Swan 2005	284	0	8	5	0.97 [0.95, 0.99]	1.00 [0.48, 1.00]
Veron 2009	187	2	1	73	0.99 [0.97, 1.00]	0.97 [0.91, 1.00]
Vo 2007	53	0	0	2	1.00 [0.93, 1.00]	1.00 [0.16, 1.00]
Waitumbi 2011	65	49	0	81	1.00 [0.94, 1.00]	0.62 [0.53, 0.71]

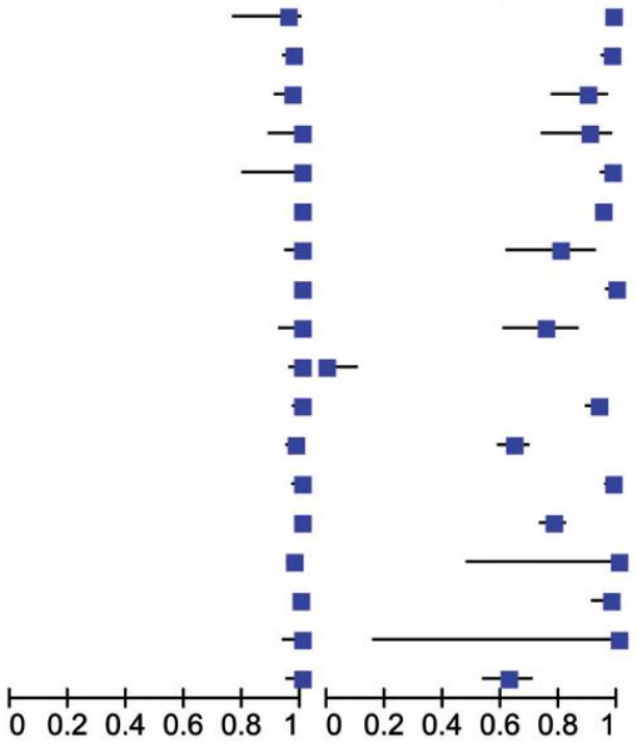


Figure 4. Forest plots of sensitivity and specificity of real-time PCR with microscopy as a reference standard. Squares represent values for sensitivity and specificity, bars show the 95% CI. *Test comparison not included in the meta-analysis, because of incomplete 2 × 2 tables. ** Two different assays were evaluated in one study; the first *P. falciparum* specific, the second *P. vivax* specific.

Sensitivity 100% Specificity 93%

Roth et al., ***Molecular malaria diagnostics: A systematic review and meta-analysis*** *Critical Reviews in Clinical Laboratory Sciences, Volume 53, 2016 - Issue 2*

Walk *et al. Malar J (2016) 15:398*
DOI 10.1186/s12936-016-1434-z

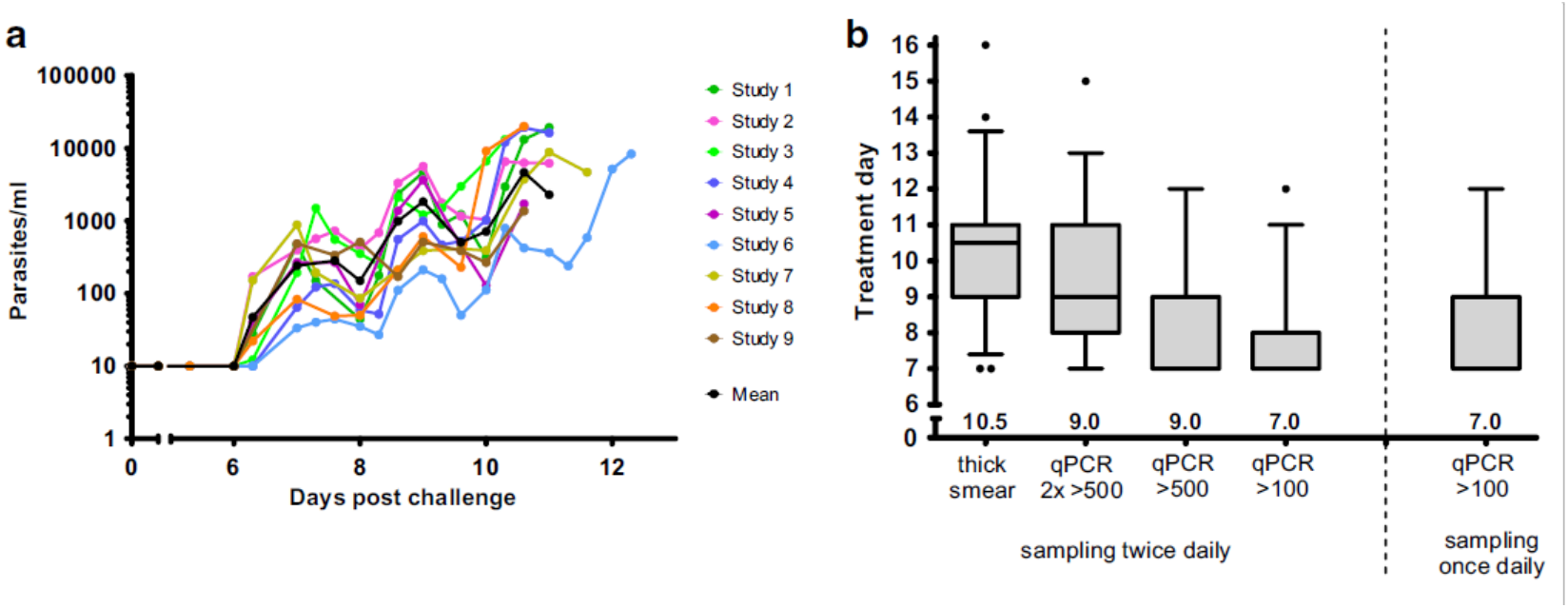
Malaria Journal

RESEARCH Open Access

Diagnosis and treatment based on quantitative PCR after controlled human malaria infection

Jona Walk[1†], Remko Schats[2†], Marijke C. C. Langenberg[2], Isaie J. Reuling[1], Karina Teelen[1], Meta Roestenberg[1], Cornelus C. Hermsen[1], Leo G. Visser[2] and Robert W. Sauerwein[1*]

qPCR with threshold 100 p/ml 100% sensitivity



Prepatent period ↓ (10,5 → 7 days)
Due to higher sensitivity of qPCR → potential earlier treatment and less complications

Malaria Journal

BioMed Central

Methodology

Open Access

Detection and identification of human *Plasmodium* species with real-time quantitative nucleic acid sequence-based amplification

Petra F Mens*[1,2], Gerard J Schoone[1], Piet A Kager[2] and Henk DFH Schallig[1]

Malaria Journal 2006, **5**:80

http://www.malariajournal.com/content/5/1/80

Table 2: Positive *Plasmodium* samples.

Species	Microscopy	NASBA *Pf*	NASBA *Pv*	NASBA *Po*	NASBA *Pm*
P. falciparum	11	11	0	0	0
P. vivax	37	0	37	1	0
P. malariae	7	3	0	0	5
P. ovale	4	0	1	4	0
Mixed infection	20	20	2	0	17
P. berghei	12	0	0	0	0

The table gives an overview of the positive results of the 99 *Plasmodium* samples analyzed with microscopy and NASBA.

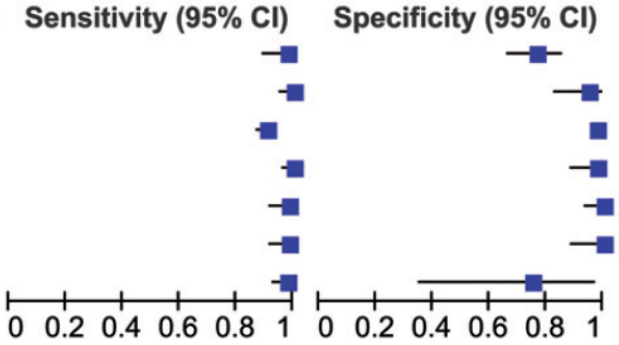
Species determination is also possible

Various molecular methods

LAMP against microscopy /PCR

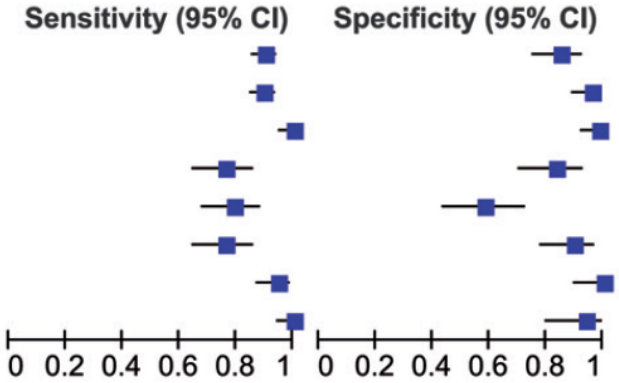
LAMP vs microscopy Sensitivity 98% Specificity 97%

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Lee 2012	46	19	1	62	0.98 [0.89, 1.00]	0.77 [0.66, 0.85]
* Poschl 2010	67	2	0	36	1.00 [0.95, 1.00]	0.95 [0.82, 0.99]
Sattabongkot 2014	244	15	25	615	0.91 [0.87, 0.94]	0.98 [0.96, 0.99]
Singh 2013	82	1	0	43	1.00 [0.96, 1.00]	0.98 [0.88, 1.00]
Sirichaisinthop 2011	59	0	1	50	0.98 [0.91, 1.00]	1.00 [0.93, 1.00]
Tao 2011	59	0	1	29	0.98 [0.91, 1.00]	1.00 [0.88, 1.00]
Yamamura 2009	87	2	2	6	0.98 [0.92, 1.00]	0.75 [0.35, 0.97]



LAMP vs PCR Sensitivity 96% Specificity 91%

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Hopkins 2013	179	11	20	62	0.90 [0.85, 0.94]	0.85 [0.75, 0.92]
** Hopkins 2013	178	3	21	70	0.89 [0.84, 0.93]	0.96 [0.88, 0.99]
Lee 2012	64	1	0	63	1.00 [0.94, 1.00]	0.98 [0.92, 1.00]
** Paris 2007	51	8	16	40	0.76 [0.64, 0.86]	0.83 [0.70, 0.93]
Paris 2007	53	20	14	28	0.79 [0.67, 0.88]	0.58 [0.43, 0.72]
** Paris 2007	51	5	16	43	0.76 [0.64, 0.86]	0.90 [0.77, 0.97]
* Poschl 2010	69	0	4	32	0.95 [0.87, 0.98]	1.00 [0.89, 1.00]
Tao 2011	57	2	0	30	1.00 [0.94, 1.00]	0.94 [0.79, 0.99]



ıre 7. Forest plots of sensitivity and specificity of LAMP with microscopy and PCR as reference standards. Squares represent values for sensitivity specificity, bars show the 95% CI. *Test comparison not included in the meta-analysis, due to high risk of bias. **Multiple evaluations per study the same assay but different extraction method or read-out, whereby studies with **were excluded from the meta-analysis. The included uations are based on a heat-treatment extraction method and a visual read-out.

Roth et al., ***Molecular malaria diagnostics: A systematic review and meta-analysis*** *Critical Reviews in Clinical Laboratory Sciences, Volume 53, 2016 - Issue 2*

Illumigene

-POCT (60 min)

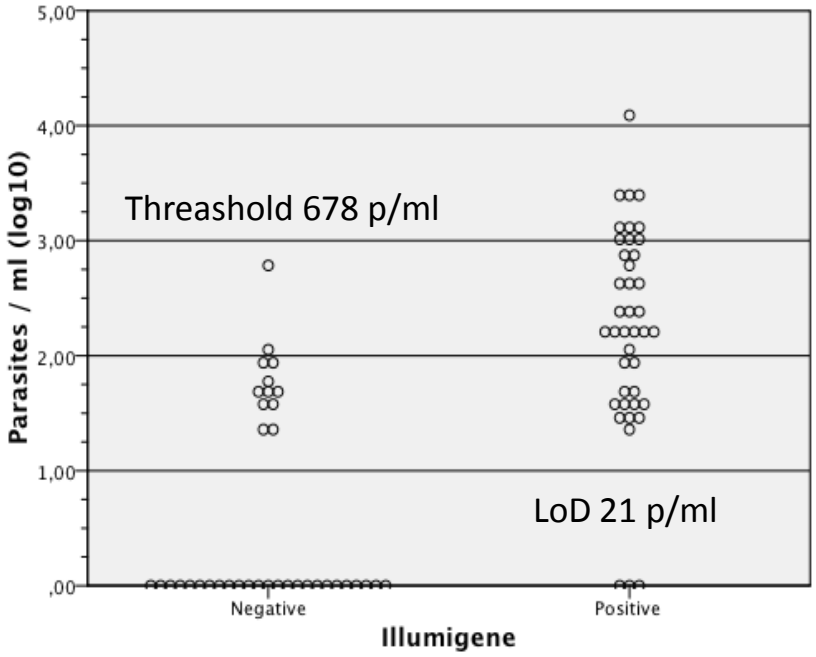
-LAMP assay

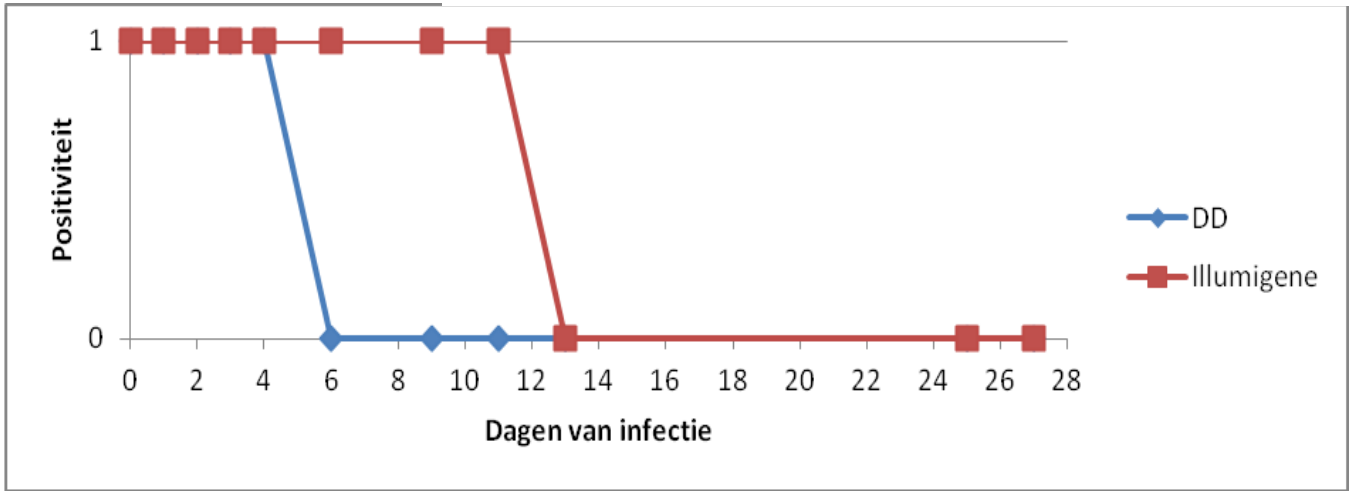
-Positive / Negative

-No differentian various plasmodia

-Our results; Se 76% & NPW 68%

-Use in follow up [1] ?





1. Jarra W, Snounou G. Only viable parasites are detected by PCR following clearance of rodent malarial infections by drug treatment or immune responses. Infect Immun 1998;66:3783–7

Direct-on-blood PCR

These assays circumvent the need for DNA extraction

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Barker 1992	157	131	41	297	0.79 [0.73, 0.85]	0.69 [0.65, 0.74]
Fuehrer 2011	89	6	0	45	1.00 [0.96, 1.00]	0.88 [0.76, 0.96]
Laserson 1994	7	1	2	38	0.78 [0.40, 0.97]	0.97 [0.87, 1.00]
Tirasophon 1994	88	32	9	461	0.91 [0.83, 0.96]	0.94 [0.91, 0.96]

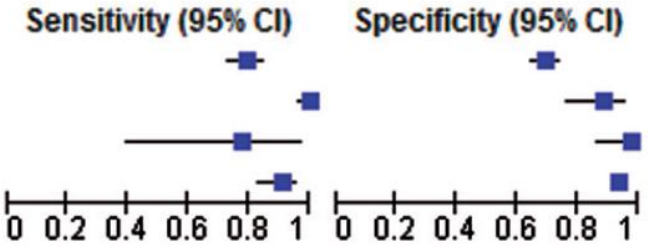
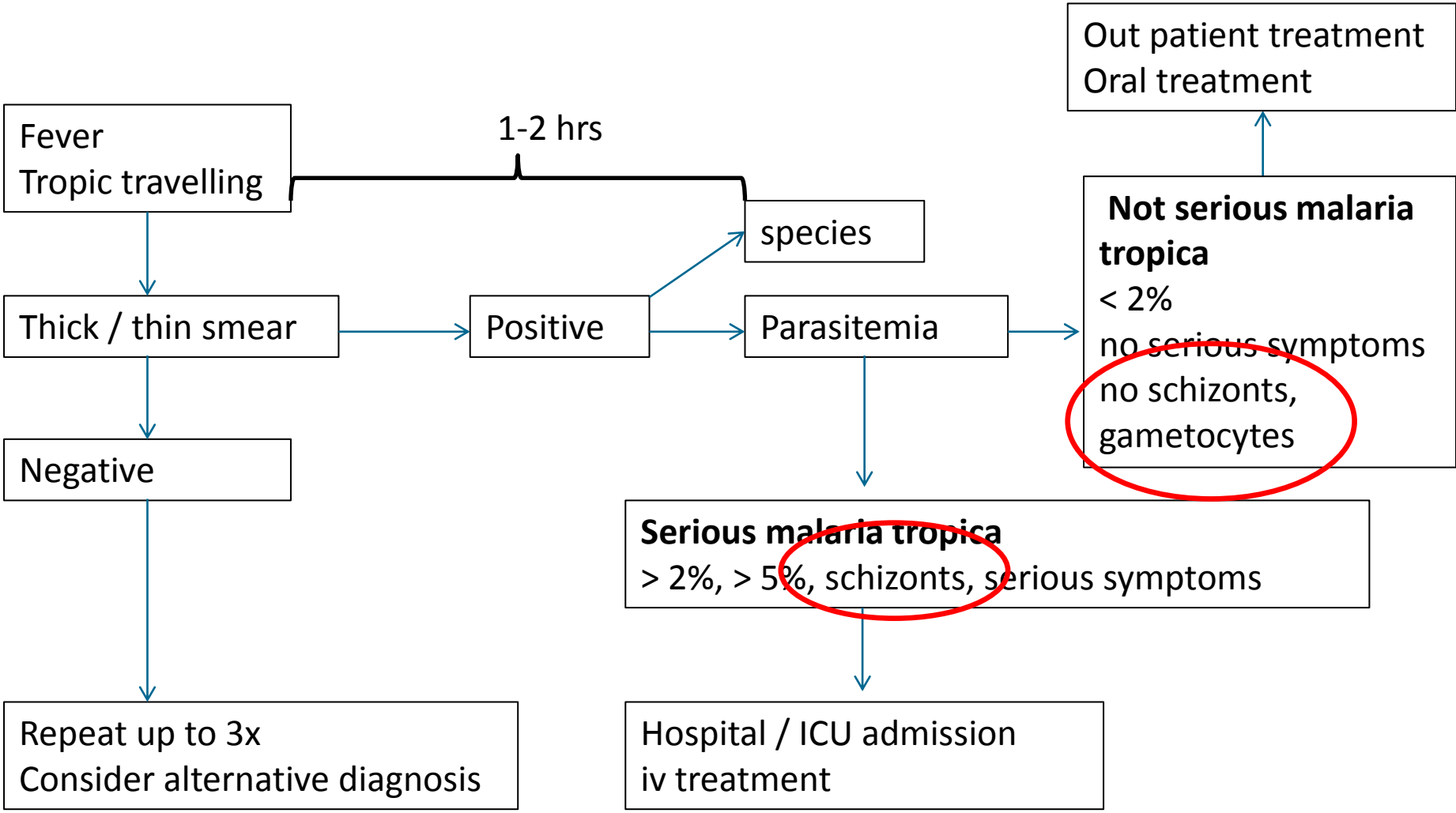


Figure 6. Forest plots of sensitivity and specificity of direct-on-blood PCR with microscopy as a reference standard. Squares represent values for sensitivity and specificity, bars show the 95% CI.

Sensitivity 93% and Specifity 90%

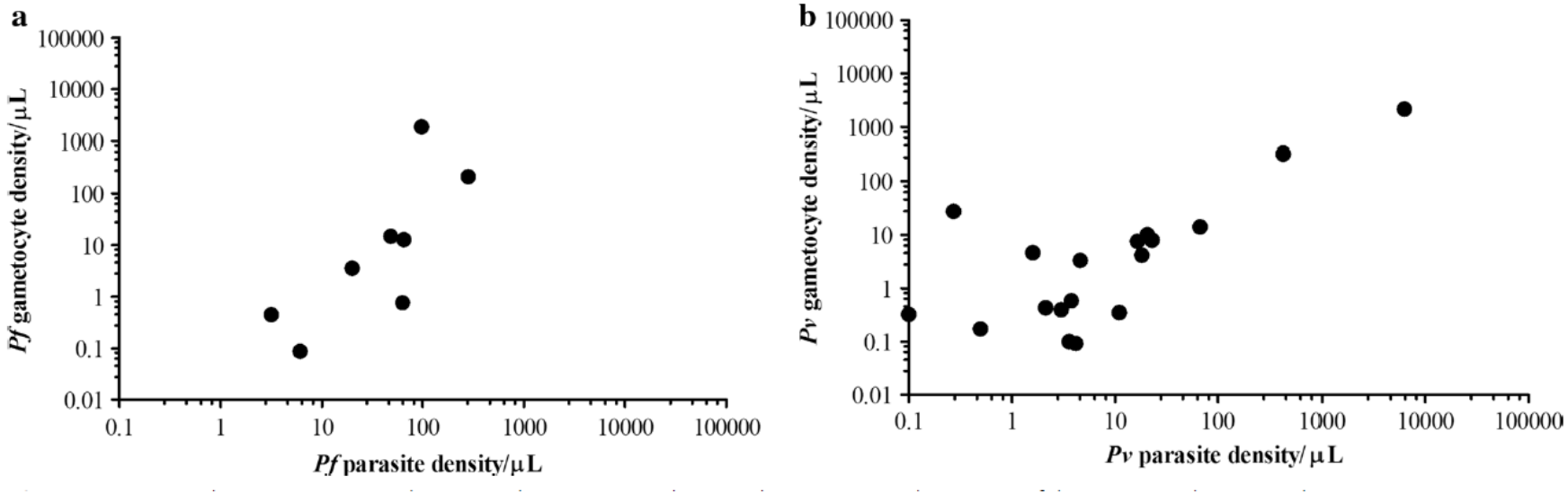
Roth et al., ***Molecular malaria diagnostics: A systematic review and meta-analysis*** *Critical Reviews in Clinical Laboratory Sciences, Volume 53, 2016 - Issue 2*

Moleculair diagnostics; what about parasite stages



Studie van Hellemond

Gametocyte densities are correlated with parasite densities



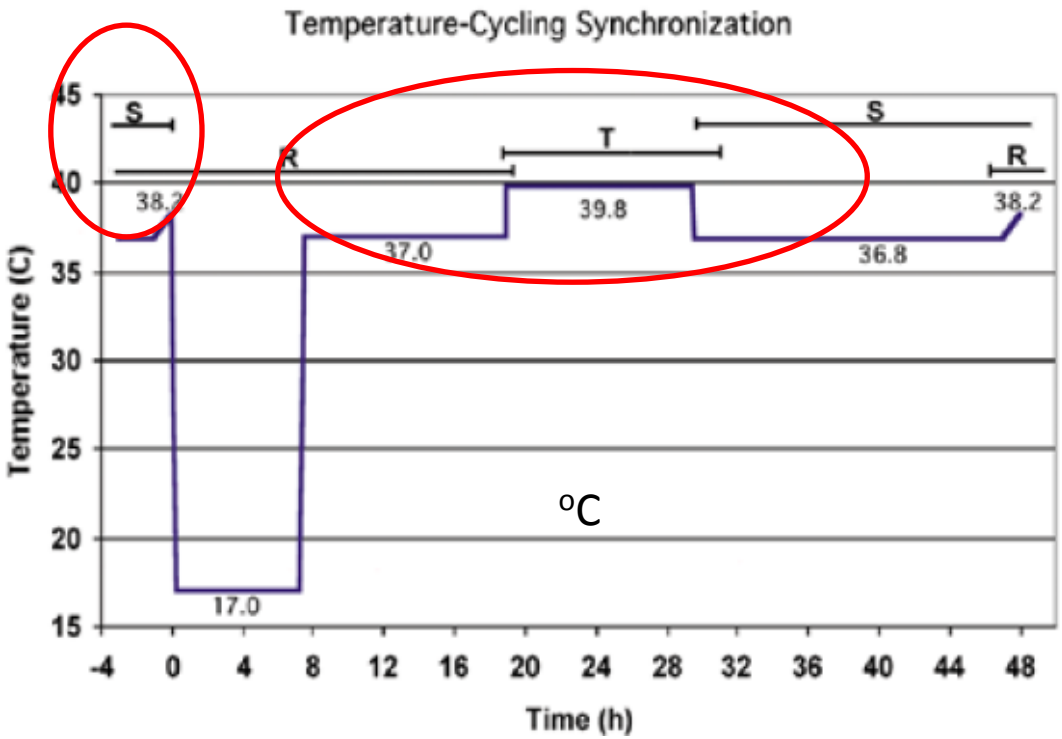
Part of what we measure by diagnostic qPCR is gametocytes.
Currently we do not differentiate.

Tadesse *et al. Malar J (2017) 16:99*

Radboudumc

What about schizonts ?

Invasion

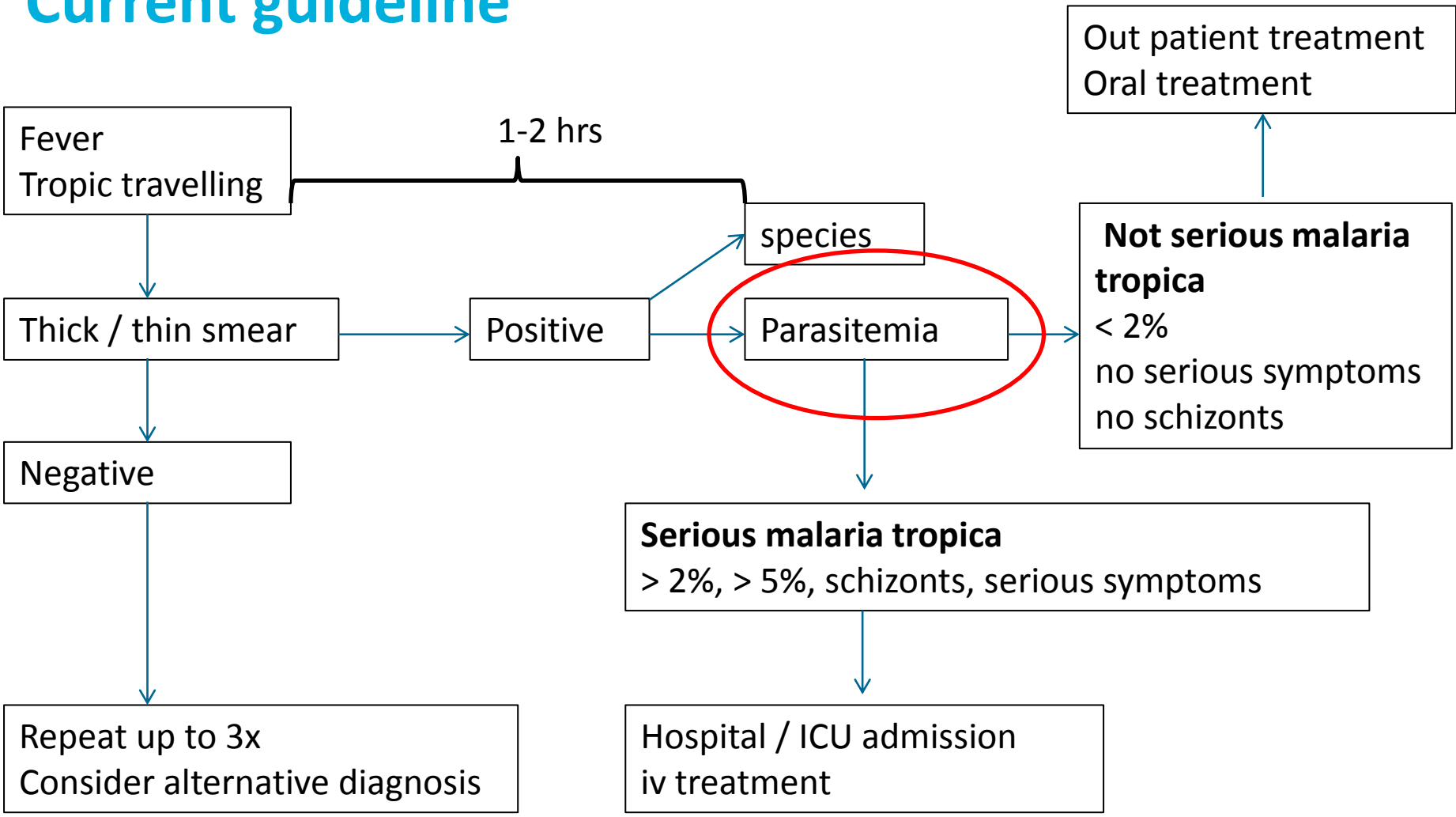


Parasite development; temperature sensitive event;
-37 °C trophozoites → schizont in 6 hour
-Temperatures of 39,8 °C and 17,0 °C prevent trophozoites development

Stage specific gene transcription during erythrocytic developmen; MAEBL and AMA-1 expression indicates schizogony

No studies in diagnostic practise, but is it important?

Current guideline



Quantification of clinical samples not evident

TABLE 2. Parasite burden ascertained by real-time PCR quantification versus microscopy

Plasmodium species	Quantification range (copies/μl)	Parasitemia range (parasites/μl)
P. falciparum[a]	$0.45–2.7 \times 10^6$	$16–1.2 \times 10^5$
P. vivax[b]	$1.10–2.5 \times 10^5$	$40–3.5 \times 10^4$
P. ovale	$57–4.8 \times 10^4$	$50–1.2 \times 10^4$
P. malariae	$1.0–1.1 \times 10^4$	$150–2.1 \times 10^3$
Mixed infections	$911–9.3 \times 10^4$	$520–6.5 \times 10^4$

[a] Significantly correlated with parasitemia ($P = 0.05$).
[b] Significantly correlated with parasitemia ($P = 0.01$).

Relation is confounded by multicopy nature of rRNA genes, variable number of these genes and multinucleated schizont stages

Farcas et al., JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2004, p. 636–638

Nog een studie over quantificering

PCR *Plasmodium spp*. Radboudumc

Two multiplex RT-PCR's:

1) MAL1(P knowlesi(CY500), P vivax(FAM), P falciparum(HEX en PhHV

2) MAL2 (P malariae CY500), P ovale curtisi (FAM) en P ovale wallikeri (HEX)

PCR is run on a Roche FLOW system

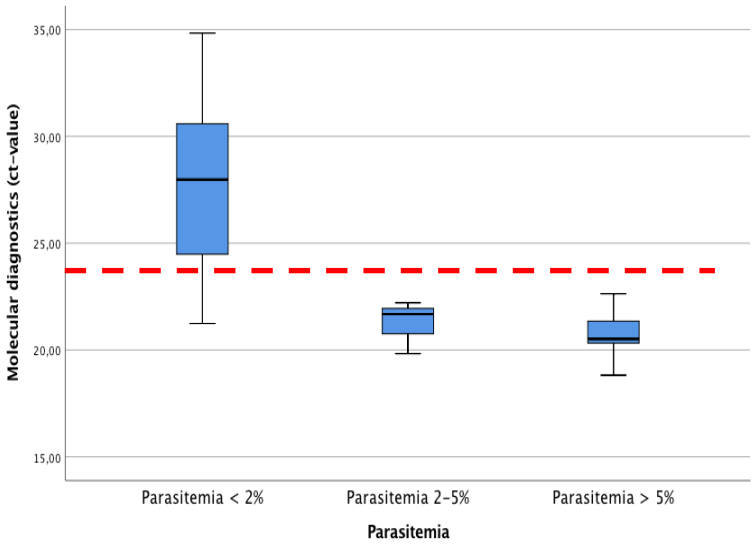
Day time routine

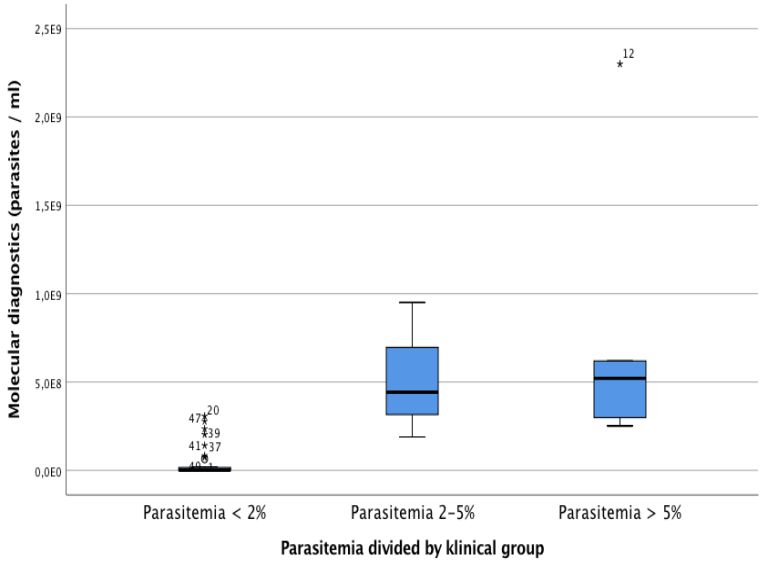
Runtime; DNA isolation 4 hrs and Amplification 2 hrs, pre and post sample handling

Threashold 20 parasites / ml

Quantitative results

Quantification of clinical samples





No information on schizonts, differentation above 2% needs a dilution step

Time to diagnosis

Microscopy
-Highly qualified technician
-Negative result 30 min
-Positive result 90 min
-Plasmodium type
-parasitemia

90 min

Antigen
-POCT
-Negative result 30 min
-Positive result 30 min
-Microscopic confirmation 90 min

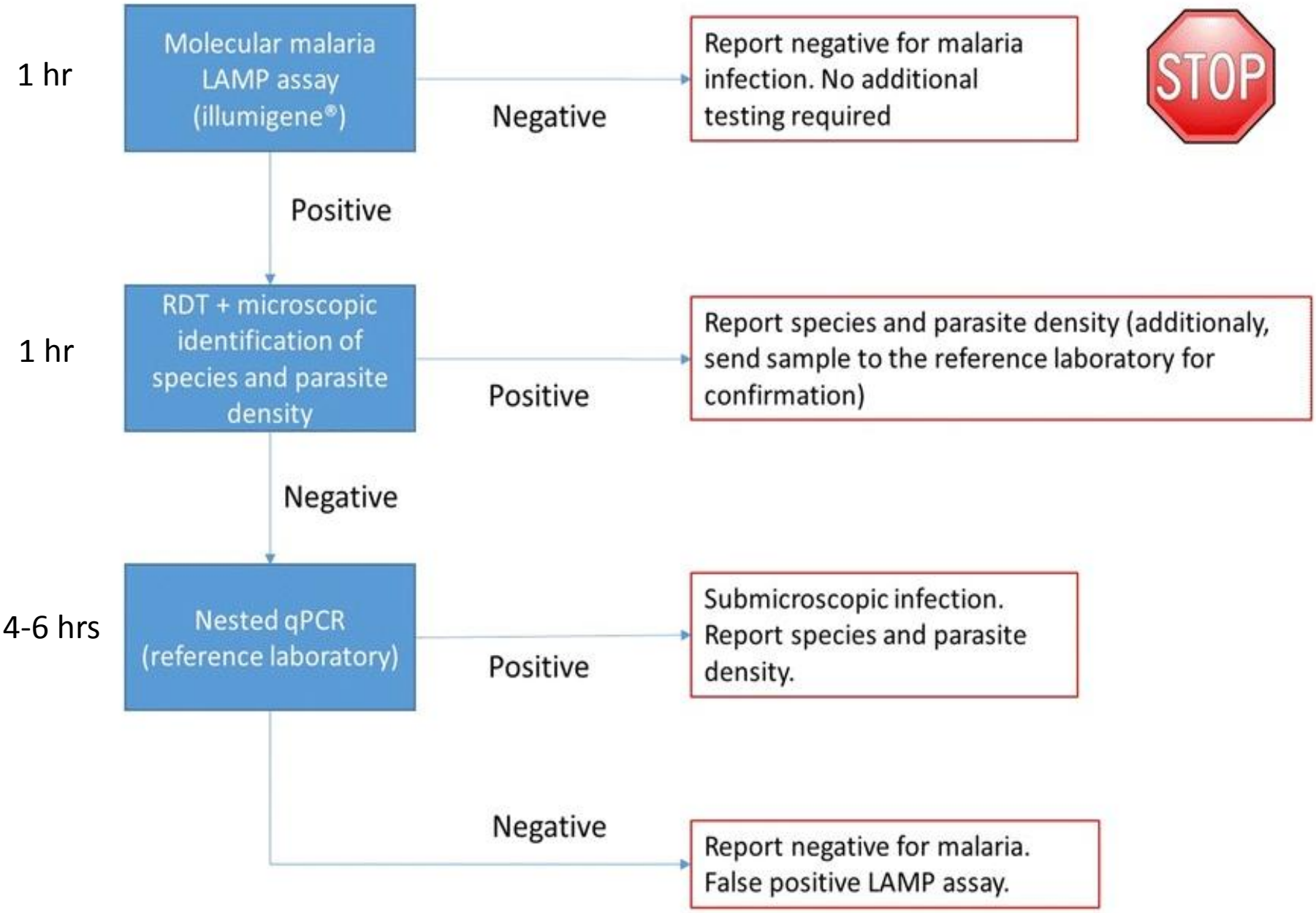
120 min

Illumigene
-POCT
-Negative result 60 min
-Positive result 60 min
-Microscopic confirmation 90 min

150 min

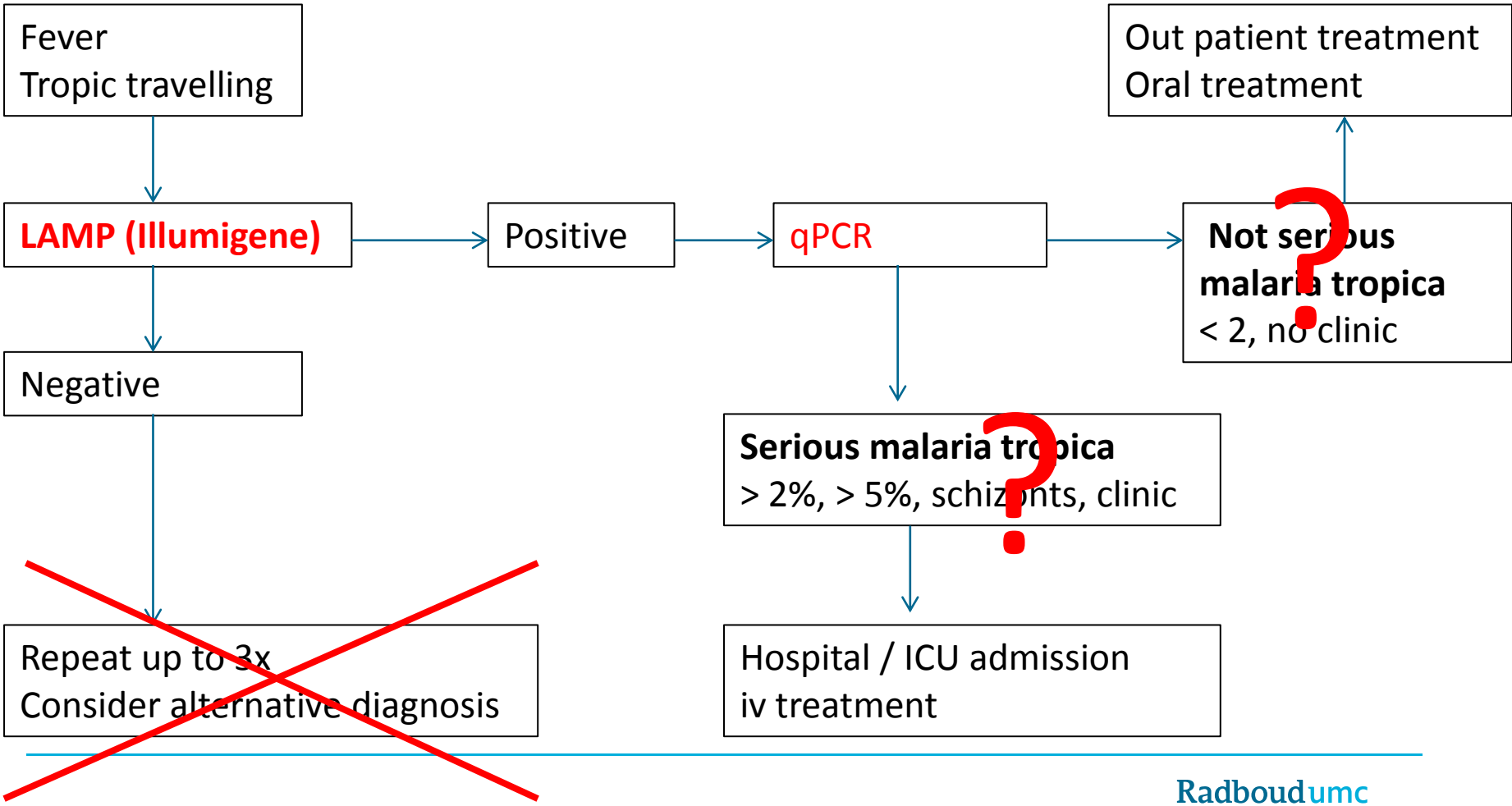
PCR
-Highly qualified technician
-Currently day-time diagnostics
-Negative result 240 min
-Positive result 240 min
-Plasmodium type
-parasitemia

240 min

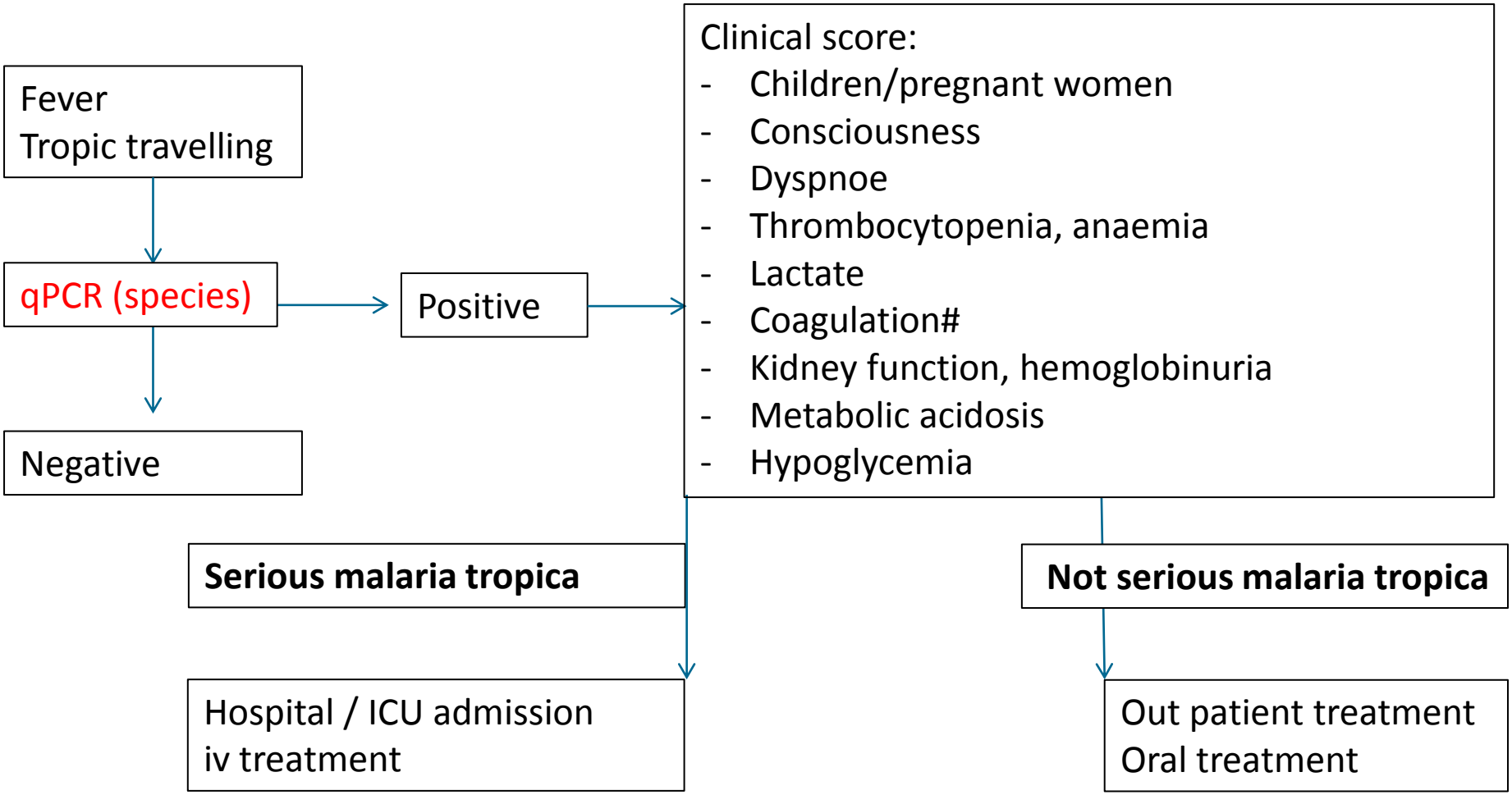


Koninck et al. , Malar J. 2017 Oct 17;16(1):418

Guideline malaria diagnostics in molecular time?



Guideline malaria diagnostics in molecular time?



Conclusions

- 18S rRNA gene was the most frequently used target
- Superior sensitivity, low LoD
- Superior sensitivity in detecting mixed infections

BUT

- Guideline in non-endemic settings will have to be adjusted
  - Clinical score
  - Quantification
  - Gametocyte markers
  - How to do follow up

→ Clinical studies are needed