Sensitivity and specificity of molecular methods for detecting markers of antimalarial drug resistance in clinical samples of *Plasmodium falciparum*: a systematic review

Rebekah Catherine Burrow

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Abstract

Background Each year infection with *Plasmodium* causes millions of clinical cases of malaria, and hundreds of thousands of deaths. Resistance to different antimalarial medications continues to develop and spread, threatening effective prophylaxis and treatment. Monitoring of resistance is required to inform health policy and preserve effective antimalarials; molecular methods can be used to determine likely parasite susceptibility. There is no consensus on the most accurate methods; large variation exists in practice. The goal of this systematic review was to identify the sensitivity and specificity of each molecular method for detecting antimalarial resistance markers.

Methods All diagnostic accuracy studies that examine at least two molecular methods for detecting selected markers of antimalarial resistance in blood samples from patients diagnosed with, or suspected of having malaria were included. MEDLINE, EMBASE, BIOSIS, and Science Citation Index were searched. Methodological quality was evaluated using QUADAS-2. Sensitivity and specificity were calculated and results synthesised and compared narratively.

Results 27,575 search results returned 18 eligible studies, examining 13 index tests, against five reference tests, for their accuracy in detecting 24 molecular markers. Some markers and tests were investigated multiple times, but rarely in the same combinations. Characteristics of studies were poorly reported. Generally, risk of bias and applicability concerns were unclear. Estimates of sensitivity and specificity were calculable for most evaluations, confidence intervals were generally wide.

Discussion Estimates of the accuracy of molecular methods are generally imprecise, contributing to uncertainty. Many methods may be close to 100% in sensitivity or specificity. Most methods require specialist equipment unlikely to be available in many low resource settings. High quality study design focusing on methods useable in low resource settings, prospective registration, and reporting according to STARD 2015 guidelines are essential for future studies.

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Dissertation Authenticity Statement

I confirm that I have read the Department of Continuing Education's rules relating to plagiarism as found in the 2016-2017 Student Handbook.

I confirm that the work presented here is my own except where otherwise indicated.

Name	Rebekah Catherine Burrow
Candidate Number	1005809
College	Kellogg College
Address	12 Ruskin Close Oxford OX2 9FU

Signed

Date 27th September 2017

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Glossary

Molecular methods

ASPCR	Allele-Specific Polymerase Chain Reaction
LAMP	Loop-Mediated Isothermal Amplification
LDR-FM	Multiplex Ligase Reaction-Fluorescent Microsphere assay
PCR	Polymerase Chain Reaction
PCR-DIG-HTA	Polymerase Chain Reaction Digoxigenin-labeled Chemiluminescent Heteroduplex Tracking Assay
PCR-HRM	Polymerase Chain Reaction with High-Resolution Melt
PCR-MSS-HTA	Polymerase Chain Reaction with Multiple Site Specific Heteroduplex Tracking Assay
PCR-PEXT	Polymerase Chain Reaction with Primer Extension
PCR-PEXT-NALFIA	Polymerase Chain Reaction with Primer Extension and Lateral Flow Immunoassay
PCR-RFLP	Polymerase Chain Reaction Restriction Fragment Length Polymorphism
PCR-SSCP	Polymerase Chain Reaction Single Strand Conformational Polymorphism
RT-PCR	Real-Time Polymerase Chain Reaction
RT-PCR-FRET/MCA	Real-Time Polymerase Chain Reaction and Melting Curve Analysis using Fluorescence resonance energy transfer
RT-PCR-HRM	High-Resolution Melt Real-Time Polymerase Chain Reaction
RT-PCR-MCA	Real-Time Polymerase Chain Reaction with Melting Curve Analysis
SSOP-ELISA	Sequence Specific Oligonucleotide Probe Enzyme Linked Immunosorbent Assay
TAC	TaqMan Array Card

Genes

pfcrt	*Plasmodium falciparum chloroquine resistance transporter*
pfcytb	*Plasmodium falciparum cytochrome b*
pfdhfr	*Plasmodium falciparum dihydrofolate reductase*
pfdhps	*Plasmodium falciparum dihydropteroate synthase*
pfk13	*Plasmodium falciparum kelch 13*
pfmdr1	*Plasmodium falciparum multi-drug resistance transporter 1*

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Introduction

Background

Infection with *Plasmodium* caused 148-304 million clinical cases of malaria, and 235-639 thousand deaths in 2015 (WHO, 2016c). Malaria is often treated effectively with an artemisinin-based combination therapy (ACT); since 2000 the incidence of cases and mortality rates have declined by 41% and 62% respectively (Sow et al., 2016, Toure et al., 2016, Pregact Study Group et al., 2016, WHO, 2016c). However, some *Plasmodium* parasites are resistant to other antimalarial chemotherapies and responsible for increased mortality (Trape et al., 1998, Korsinczky et al., 2000, Nosten et al., 2000, Bousema et al., 2003, Mullick et al., 2011).

ACTs and artemisinins are the World Health Organization (WHO) recommended first-line treatments for most patients with uncomplicated *falciparum* malaria, or with severe malaria (WHO, 2015b). However, resistance to artemisinins has recently emerged in the Greater Mekong sub-region, linked to mutations in the *plasmodium falciparum kelch 13* (*pfk13*) gene, with a phenotype of delayed parasite clearance which may lead to treatment failure (Ariey et al., 2014, Ashley et al., 2014, Tun et al., 2015, Amaratunga et al., 2016, Thanh et al., 2017, Imwong et al., Chang et al., 2016). Resistance to ACTs may be evolving in other regions but failure of ACTs may also be due in part to other factors, such as resistance to partner drugs (Venkatesan et al., 2014, Lu et al., 2017, Mukherjee et al., 2017).

Efforts to eliminate malaria have been intensified in the Greater Mekong sub-region and world-wide surveillance of artemisinin resistance has been prioritised (WHO, 2016b, Sesay et al., 2017) .

However, a minority of patients with malaria receive WHO recommended ACTs; most patients receive either no antimalarial drug, or a different antimalarial, to which resistance (with a phenotype of treatment failure) may already be widespread (Littrell et al., 2014, WHO, 2016c, WWARN, 2016, ACTwatch Group et al., 2017b). Chemoprevention for high-risk groups in endemic countries often uses a combination of non-artemisinins; increasing resistance threatens efficacy (Meremikwu et al., 2012,

Geiger et al., 2014, Muanda et al., 2015, Cisse et al., 2016). For travellers to endemic countries, chemoprevention is recommended. Resistance to mefloquine has already developed (Price et al., 2004). Although some cases of resistance to atovaquone have been reported, resistance has not spread (Jacquerioz, 2015, Goodman et al., 2016). Mass drug administration (MDA) programs have been used with mixed success in the past, and are currently being trialled. The WHO recommendations are to use a drug combination for MDA different to that for first-line treatment in order to try to preserve antimalarials (Poirot et al., 2013, University of Oxford, 2013, WHO, 2015c).

Effective prophylaxis and treatment are essential for control of malaria and eventual elimination, but the emergence and spread of drug resistant parasites is a great impediment to achieving these goals. In the future, diagnosis and treatment will be supplemented by add-on point-of-care tests for detection of antimalarial resistances, as it is for other infectious diseases (Steingart et al., 2014, Krampa et al., 2017, Roth et al., 2016). Until these diagnostics are developed and implemented widely to allow individualised treatment, surveillance will remain key to determining the chemoprevention and treatments most likely to be effective. Continuous global monitoring of therapeutic efficacy and drug resistance is required to detect epidemiological patterns of parasite susceptibility to all antimalarial drugs in order to inform malaria prevention and treatment policies (Plowe, 2003, Sibley et al., 2008, WHO, 2010, Moss et al., 2015, WHO, 2015a).

In addition to identifying emerging resistance, and altering policy to ensure that effective antimalarials are recommended, surveillance can also detect reductions in resistance. Fitness costs incurred by parasites with mutations conferring resistance, and antimalarial drugs exerting opposite selective effects, will contribute to the re-emergence of parasites sensitive to previously discarded antimalarials, which can then be redeployed (Ord et al., 2007, Humphreys et al., 2007, Sisowath et al., 2009, Kublin et al., 2003, Laufer et al., 2006, Lucchi et al., 2015). Reducing the use of ACTs by utilising other effective antimalarials, should slow the emergence and spread of resistance to ACTs and preserve them as effective antimalarials (Dondorp et al., 2010, Maude et al., 2010, Klein, 2013).

Historically, parasite susceptibility was determined using clinical trials or observational studies. In addition, for a limited number of clinical samples, therapeutic efficacy could be tested through *ex vivo* and *in vitro* culture methods. More recently developed molecular methods offer many advantages over both of these; reduced patient burden, reduced requirement for staff with clinical, trial and laboratory expertise, reduced delay to results, and increased throughput (Scientific Group on Resistance of Malaria Parasites to Drugs, 1965, WHO, 1973, Kublin et al., 1998, Plowe, 2003, Abdul-Ghani et al., 2014).

Molecular methods are able to detect single nucleotide polymorphisms (SNPs) and copy number variation (CNV) associated with resistance of *P. falciparum* to many antimalarial drugs. The correlation between genotype and phenotype is mixed in strength for different molecular markers, so the clinical relevance of these molecular markers varies.

For example, when the T76K mutation is present in the *chloroquine resistance transporter* (*pfcrt*) gene, almost all patients fail to respond adequately to chloroquine treatment (Wellems and Plowe, 2001, Ecker et al., 2012). However, combinations of mutations in the *dihydropteroate synthase* (*pfdhps*) and *dihydrofolate reductase* (*pfdhfr*) genes are sometimes highly predictive of sulphadoxine-pyrimethamine (SP) treatment failure (East Africa) and sometimes not (West Africa) (Mockenhaupt et al., 2005, Alker et al., 2008, Mbacham et al., 2010, Warsame et al., 2017).

Factors other than one mutation affect the presence and level of resistance, including other known mutations (such as *pfcrt* 72S in combination with 76K), as yet unknown genetic mutations conferring some degree of resistance directly, other parasite characteristics (including parasitaemia and virulence), host immunity, and interactions between parasite and host (Sherman, 1998, Grech et al., 2006, Schneider et al., 2012, Klein, 2013). In spite of these issues, genotyping of antimalarial resistance markers is the surveillance method used most often and by most research groups (WWARN, 2017).

There is a lack of a standardised protocol for detecting resistance via molecular markers, with variation in sample source, collection and storage, DNA extraction, methods for DNA amplification, detection,

and analysis of results of mutations associated with resistance (Jones et al., 2012, WHO, 2010, Bauer et al., 2003, Taylor et al., 2011, Felger et al., 2003, Wilson et al., 2005a). Attempts have been made by organisations such as the WorldWide Anti-malarial Resistance Network (WWARN) to standardise some methods including collection of blood, DNA extraction, and RT-PCR for *pfmdr1* copy number. However, these have been done without ascertaining the optimum methods through a systematic review of the available diagnostic accuracy studies (WWARN, 2014).

It seems likely that there is variation in the accuracy of different molecular methods for detecting antimalarial drug resistance. For example, Ndiaye et al. (2013) reported determining *pfdhfr* N51I, C59R and S108N for 416/416 (100%) of samples, while Ojurongbe et al. (2011) determined a genotype for 39/79 (49%) of samples. Some differences may be due to patient and sample characteristics, such as parasitaemia, but it seems likely that test performance also has a key role.

Using substandard methods to detect resistance is wasteful, requiring more resources for the generation of less data, and less accurate data. Using substandard methods may produce actively misleading results. It does not respect the contribution made by the thousands of research participants who donate to us their personal data, time and blood, and who are the most in need of effective surveillance programs. It unnecessarily uses some of our limited resources that could be used more effectively elsewhere in malaria programs.

If surveillance efforts fail to detect the presence of anti-malarial resistance, or detect a much lower level than is present, treatment guidelines may advise the use of antimalarials that are ineffective for many patients, increasing the number of cases and deaths. If surveillance is not sufficiently sensitive to detect small changes in anti-malarial resistance it will not be able to detect either returning sensitivity or emerging resistance. Detection of emerging resistance in particular is critical to controlling the spread of resistance and preserving the utility of existing antimalarials.

Rationale

Although many systematic reviews have been carried out for diagnostic tests for infectious diseases, few have been carried out examining accuracy of drug resistance markers, and none in malaria (Steingart et al., 2014, Leeflang, 2014, Verbakel et al., 2017). This systematic review is the first of its kind, to systematically collate and compare estimates of test sensitivity for detection of antimalarial resistance markers in malaria. This systematic review aims to:

- create the first comprehensive list of molecular methods for genotyping drug resistance markers that have been examined in diagnostic test accuracy studies
- collate the sensitivity and specificity of each of the molecular methods for each of the markers of antimalarial resistance
- allow researchers to identify which may be the most accurate methods for them to use with the resources available to them
- allow an analysis of the gaps in published research, to highlight:
    - which techniques may require further studies of diagnostic test accuracy
    - identify gaps in the evidence base for detection of specific important molecular markers
    - where studies with a lower risk of biases and greater applicability may be required

This will improve the efficiency of research, requiring the use fewer resources to successfully identify antimalarial markers of interest. As the research community adopts more sensitive and specific methods and discards less accurate methods, the impact will be evident as fewer gaps in surveillance, and improved accuracy and precision of surveillance. This improved coverage will improve the quality of evidence of antimalarial resistance epidemiology by increasing the accuracy and precision of estimates of known resistance. It will enable the mapping of more localised patterns of resistance and improve our ability to detect smaller changes in patterns.

The higher quality evidence generated will be used, hopefully, to inform guidelines on the use of antimalarial drugs, guidelines based on this more complete, accurate and precise information are likely to be of higher quality themselves, and lead to better outcomes for more patients. In order to assess any impact on patient outcomes, further studies would be required (Ferrante di Ruffano et al., 2012).

Objectives

The objectives of this review are, for diagnostic test accuracy studies, examining blood samples taken from symptomatic or asymptomatic patients known to be, or suspected of carrying *Plasmodium falciparum* parasites, and comparing at least two molecular methods for determining genotypes of antimalarial drug resistance markers to:

- Obtain per study, estimates of the sensitivity and specificity of each molecular method for determining the genotype of each drug resistance marker.
- Obtain summary estimates of the sensitivity and specificity of each molecular method for determining the genotype of each drug resistance marker.
- Use statistical methods to compare estimates of sensitivity and specificity of different methods to identify the most sensitive and specific methods.
- Narratively synthesise and compare estimates of sensitivity and specificity to identify the most sensitive and specific methods when statistical synthesis and comparison are not appropriate.

The samples will be from natural infections, i.e. not from membrane feeding studies, spiked *in vitro* or from *in vitro* cultures. The index and reference tests will include any molecular methods for determining the genotype of at least one of the molecular markers selected in this review. An exhaustive list of molecular methods will not available until after this review is completed. Anti-malarial resistance markers will be chosen when:

- an individual SNP, alone or in combination with other SNPs, have been shown to be associated with treatment failure
- *in vitro* experiments have shown a phenotype of resistance
- emerging evidence shows that markers are likely to be of interest in future surveillance

Methods

This systematic review protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO) on 30th January 2017 and was last updated on 28th July 2017 (registration number CRD42017056249) (CRD, 2016). This systematic review was designed and reported using PRISMA-P, PRISMA and STARD guidance, the PRISMA checklist is available in the appendices (Moher et al., 2009, Moher et al., 2015, Shamseer et al., 2015, Bossuyt et al., 2015).

Eligibility criteria

Studies were selected according to the following eligibility criteria.

Inclusion criteria:

- diagnostic accuracy studies, including case-control studies
- studies examining any number of samples
- studies carried out in any setting
- studies including samples taken from patients or asymptomatic patients when there is a suspicion that they may be carrying *Plasmodium* parasites
- studies comparing at least two molecular methods
- determining the genotype of at least at least one molecular marker listed in Table 1
- where sufficient data to construct a 2x2 table of test accuracy can be obtained
- studies with reports published in any language
- studies for which there are published reports

Exclusion criteria:

- Studies reported before 1983
- Data generated using lab-adapted *Plasmodium* strains and 'spiked' samples

Table 1. Selected molecular markers of antimalarial resistance

gene	amino acid	wildtype	mutant	associated with sensitivity or resistance to:
pfcrt (Djimde et al., 2001, Beshir et al., 2010, Picot et al., 2009, Baraka et al., 2015, Otienoburu et al., 2016, Some et al., 2010)	72	C	S	amodiaquine, chloroquine, lumefantrine, piperaquine
	74	M	I	
	75	N	E	
	76	K	T	
plasmodium falciparum cytochrome b (pfcytb) (Musset et al., 2006, Sutherland et al., 2008)	268	Y	N/S/C	atovaquone
pfdhfr (Gadalla et al., 2013, Picot et al., 2009, Warsame et al., 2015)	16	A	V	pyrimethamine
	51	N	I	
	59	C	N	
	108	S	D	
	164	I	L	
pfdhps (Brooks et al., 1994, Reeder et al., 1996, Triglia et al., 1997, Gregson and Plowe, 2005, Menard et al., 2006, Picot et al., 2009, Gadalla et al., 2013, Warsame et al., 2015)	436	S	A/F	sulphadoxine
	437	A	G	
	540	L	E	
	581	A	G	
	613	T	S	
pfk13 (Amaratunga et al., 2016, Phyo et al., 2016, WHO, 2016c, WHO, 2016a)	458	N	Y	artemisinins
	493	Y	H	
	539	R	T	
	543	I	T	
	561	R	H	
	580	C	Y	
Plasmodium falciparum multi-drug resistance 1 (pfmdr1) (Djimde et al., 2001, Picot et al., 2009, Some et al., 2010, Otienoburu et al., 2016, Phyo et al., 2016, Sondo et al., 2016)	86	N	Y	amodiaquine, chloroquine, lumefantrine, mefloquine

Only diagnostic accuracy studies were chosen as this was considered to be the only situation in which two molecular methods would be compared. This review was intended to include as much potentially useful information as possible, so included studies carried out in any setting and of any size. In real clinical scenarios patients suspected or known to be carrying Plasmodium parasites may be tested, so both groups were included in this review. Non-molecular methods for determining antimalarial

resistance, such as culture assays, exist but measure an *in vitro* phenotypic response, not a genotype, and so cannot be used to determine the accuracy for detecting molecular markers. All index and reference tests were included as all tests were of interest and a gold standard has not been established (Rutjes et al., 2007, Reitsma et al., 2009, Naaktgeboren et al., 2013).

Ideally this review would include all molecular markers of interest, but resource constraints and the design of the search terms using the genes of interest made this impractical. Instead, markers with the strongest evidence and greatest clinical relevance were included, and markers less likely to have been studied in diagnostic accuracy studies were excluded. This review excluded studies where 2x2 tables of test accuracy could not be constructed in order to ensure that sensitivity and specificity with 95% confidence intervals (CI) could be correctly calculated in a unified manner and, where possible, take into account indeterminate results. This review was intended to include as much potentially useful information as possible, so included studies published in any language (Juni et al., 2002). Only studies for which a report was published as a conference abstract or as an article in a scientific journal were included to create an objective and replicable system for including studies.

PCR was first developed in 1983, as most molecular methods will be PCR-based, this year was used as a cut-off for inclusion. Only samples from natural infections in human patients were included to maximise the applicability of test accuracy results to patients (White et al., 2016a, Duffy and Avery, 2017, Ribacke et al., 2013, Assefa et al., 2014, van Schalkwyk et al., 2013).

Information sources

Medical Literature Analysis and Retrieval System Online (MEDLINE) via the PubMed interface, Excerpta Medica database (EMBASE) via the OVID interface, BioSciences Information Service of Biological Abstracts (BIOSIS), and Science Citation Index via Web of Knowledge were searched from inception (Hartling et al., 2016, Whiting et al., 2008, Booth, 2010, Ross-White and Godfrey, 2017). The reference lists of eligible studies identified through this search were also searched (Horsley et al., 2011). A list of the eligible studies was circulated to authors of included reports and to experts in malaria diagnostics and anti-malarial drug resistance to identify any omissions that could be remedied.

Search strategies were developed following guidance from Higgins and Green (2008), CRD (2009), and de Vet (2008), using text words and Medical Subject Headings (MeSH) terms, and checked with an information specialist. An initial search was made on 19th January 2017, and a second search on 11th August 2017. The search strategy was slightly unconventional, in the absence of a defined reference standard and index test(s) of interest; the patients and target condition were the only terms that could be used to design the search. It was planned that the search strategy be sensitive rather than specific.

The MEDLINE search strategy is included in Table 2, this was adapted to the syntax and medical subject headings of the other databases. No filters were used (Ritchie et al., 2007, Geersing et al., 2012). EndNote 7.7.1 software was used to manage literature search results.

Table 2. MEDLINE search strategy

Patients	"plasmodium falciparum"[MeSH Terms] OR "plasmodium falciparum"[Text Word]
Index Test	-
Reference Test	-
Target Condition	**AND**
	resist* [Text Word]
	AND
	"chloroquine"[MeSH Terms] OR “chloroquine”[Text Word] OR “CQ”[Text Word] OR “crt”[Text Word] OR “pfcrt”[Text Word]
	OR
	"multidrug resistance protein 1"[Text Word] OR “pfmdr1”[Text Word] OR “mdr1”[Text Word]
	OR
	"tetrahydrofolate dehydrogenase"[MeSH Terms] OR “pfdhps”[Text Word] OR “dhps”[Text Word] OR “dihydrofolate reductase”[Text Word]
	OR
	"dihydropteroate synthase"[MeSH Terms] OR “dihydropteroate synthase”[Text Word] OR “pfdhfr”[Text Word] OR “dhfr”[Text Word]
	OR
	“pfk13” [Text Word] OR “Kelch 13”[Text Word] OR “K13”[Text Word]
	OR
	"cytochromes b"[MeSH Terms] OR “pfcytb”[Text Word] OR "cytochrome b"[Text Word] OR “cyt-b”[Text Word] OR “cytb”[Text Word]

Selection process

Search results were assessed using the screening form in Figure 1. Titles were screened, and abstracts obtained for any potentially eligible studies. Abstracts were screened, and full text of reports obtained for any potentially eligible studies. Study and report details were used to identify multiple reports of the same study or multiple studies using the same samples. One report of each study was included, and duplicate reports excluded. Multiple studies potentially using the same samples were all included in this review. The first reason for ineligibility that was identified was recorded. It had been planned that a second reviewer would screen 10% of all unique search results and 100% of those judged eligible by the first reviewer. Due to the volume of work this entailed, it was not reasonable to ask a second reviewer to carry out screening in the time available. This may have impacted the quality of the review; eligible studies may have been excluded in error; Edwards et al. (2002) found that single reviewers missed between 0-24% of eligible reports compared to two reviewers when checking 100% of results, however, this may not be applicable to screening for diagnostic test accuracy studies.

Figure 1: Screening tool

Review Author Details	Date screened by Reviewer 1	
	Date screened by Reviewer 2	
	Report screened	
Report Details	Study ID (Trial Registration)	
	Report ID (PMID)	
	Author List	
	Date Published	
	Title	
Inclusion Criteria	Diagnostic accuracy study design, including case-control	
	Examining clinical blood samples taken from patients or asymptomatic patients when there is a knowledge or suspicion that they may be carrying *Plasmodium* parasites	
	Patients or samples in or from countries where *Plasmodium falciparum* is responsible for >90% of confirmed cases with or without speciation	
	Patients or samples in or from countries where *Plasmodium falciparum* is responsible for <90% of confirmed cases with speciation	
	Examining at least one of the antimalarial resistance markers:	
	o *pfcrt* (72, 74, 75, 76)	
	o *pfmdr1* (86, 184, 1034, 1042, copy number)	
	o *pfcytb* (268)	
	o *pfdhps* (436, 437, 540, 581, 613)	
	o *pfdhfr* (16, 51, 59, 108, 164)	
	o *pfk13* (458, 493, 539, 543, 561, 580)	
	Comparing any two or more molecular methods for detecting an antimalarial resistance marker	
	Providing sufficient data to construct a 2x2 table of test accuracy	
Exclusion criteria	Published before 1983	
	Including lab-adapted *Plasmodium* strains and 'spiked' samples	
	No published report	

Data collection process

Data were extracted for all eligible studies using the data extraction form in Figure 2. Results data were extracted directly into 3x3 tables for test accuracy results, illustrated in Figure 3. It had been planned that a second reviewer would independently extract 100% of the data from all eligible studies. Due to the volume of work this entailed and time limitations only one reviewer carried out data extraction; this may have impacted the quality and conclusions of the review (Day et al., 1998, Buscemi et al., 2006, Gotzsche et al., 2007, Jones et al., 2005).

The review author was not blind to the journal titles, study authors, or institution (Berlin, 1997).

For published studies seemingly eligible other than for the availability of results to construct a 2x2 table, unpublished results were sought from report authors. An email including a reply form with pre-specified data items was used (Young and Hopewell, 2011). Where data could not be obtained by 11th August 2017, studies were excluded from the analysis and listed in Table 85.

Including unpublished data in this review may have increased the amount of data, and reduced the effect of publication bias (van Enst et al., 2014, Korevaar et al., 2016, Hopewell et al., 2007). Including unpublished data may have introduced lower quality data; unpublished data has not been subject to peer review. However, for most of the studies in this review a peer-reviewed report has been published; it seems unlikely that additional data from the same study would be of a significantly lower standard, but it may introduce bias (Scherer et al., 2007).

Figure 2: Data extraction tool

Review Author Details	Date extracted by Reviewer 1	
	Date extracted by Reviewer 2	
	Request further details from authors?	
Report Details	Study ID (Trial Registration)	
	Report ID (PMID)	
	Author List	
	Date Published	
	Title	
Study	Country of study	
	Date of study	
	Type of laboratory	
Patients	Country of patient screening or diagnosis	
	Date of patient screening or diagnosis	
	Screening / active case finding	
	Diagnosis	
	Eligibility criteria	
	Sex	
	Age	
	Concomitant conditions/infections	
	Sampling method	
	Number of patients	
Samples	Country of *Plasmodium* infection	
	Date of *Plasmodium* infection	
	Source of blood (venous, 100ul, coagulant)	
	Parasitaemia	
	Speciation	
	Multiplicity of infection	
	Storage	
	Number of samples	
	Sampling method	
Index Test	DNA extraction	
	Genotyping conduct	
	Genotyping interpretation	
Reference Test	DNA extraction	
	Genotyping conduct	
	Genotyping interpretation	
Target condition	Genes and SNPs	
Flow and timing	Uninterpretable / indeterminate / missing results	
Blinding	Blinding of participants, clinicians, outcome observers or analysts	
Comparative	Interval between index and reference tests	
COI	Funding and COI	
Other	Notes	

Figure 3: 3x3 template for extraction of results

Reference Test:

Index Test:

Gene:

Sample no.		Reference Test		
		Mutant	Wildtype	Unknown
Index Test	Mutant	a	b	g
	Wildtype	c	d	h
	Unknown	e	f	i

Data items

Study and report details were extracted, and a full list of data items is described further in the review protocol in the appendices and listed in Tables 2 and 3. Microsoft Excel 2013 was used to store data.

Where haplotypes were reported, individual genotypes were extracted if possible. Where mixed sensitive and resistant antimalarial markers were described they were counted as resistant genotypes, as the presence of any proportion of resistant alleles confers a resistant phenotype. Sensitivity and specificity were calculated from the outcomes reported, or using unpublished 2x2 or 3x3 tables provided by study authors.

Risk of bias in individual studies

Risk of bias was assessed using the adapted QUADAS-2 tool in Figure 4 (Whiting et al., 2011, Hirji, 2009, Whiting et al., 2013, Reitsma, 2009). It had been planned that a second reviewer would independently assess the risk of bias and applicability concerns. Instead, due to time constraints only one reviewer carried out assessment, this may have impacted the quality of the assessment of risk of bias and applicability (Guo, 2013). The risk of bias for each domain was rated as “low”, “high”, or “unclear”. When insufficient data were available the risk of bias was rated as “unclear” (Whiting et al., 2005, Ochodo et al., 2014). RevMan 5.3 was used to graph the risk of bias and applicability concerns.

Figure 4: Modified QUADAS-2 tool

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	
	Was a case-control design avoided? Was a range of parasitaemias used?	
	Did the study avoid inappropriate exclusions?	
	Risk of bias: Could the selection of patients have introduced bias?	
	Applicability: Are there concerns that the included patients and setting do not match the review question?	
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	
	Were the reference standard results interpreted without knowledge of the results of the index tests?	
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	
	Did all patients receive the same reference standard?	
	Were all patients included in the analysis?	
	Risk of Bias: Could the patient flow have introduced bias?	

Data Synthesis

Stata 14 was used to calculate sensitivity and specificity and exact binomial 95% CI (Clopper and Pearson, 1934). Data were synthesised through narrative, following guidance developed by Popay (2006). Positive predictive values (PPV), negative predictive values (NPV) and likelihood ratios (LR) were not calculated as these are affected by prevalence. Studies in this review are not limited by setting or design; prevalence will vary hugely and predictive values and likelihood ratios will not be applicable in many scenarios.

Confidence in cumulative estimate

It had been planned that the quality of evidence would be assessed using Grading of Recommendations Assessment, Development and Evaluation (GRADE) methodology (Schunemann et al., 2008, Schunemann et al., 2016). However, application of GRADE to diagnostic accuracy studies is difficult. Given the absence of information about study characteristics and the results of the QUADAS-2 assessment of bias for most studies in this review, GRADE could not be applied in a way that met the required criteria, and would not be a useful way of assessing the evidence (Gopalakrishna et al., 2014, Schünemann, 2016, Trenti et al., 2016). The outcomes of these studies (test accuracy for detecting markers of antimalarial resistance) are removed from patient important outcomes; genotypic markers of antimalarial resistance in blood samples are variably predictive of phenotypic resistance to antimalarials in patients, the phenotype of resistance in patients is correlated with an increased chance of unfavourable patient outcomes such as severity of disease, and morbidity and mortality (Picot et al., 2009).

Studies within reviews (SWARs)

SWAR 6: Measuring the time taken for activities in systematic reviews

Data collection for SWAR 6 was started but abandoned (Burrow, 2016).

SWAR: Finding eligible studies efficiently

A proposed new SWAR is outlined in the appendices. After all eligible studies were identified, the search, slightly modified to remove duplication in searching via Web of Knowledge, was re-run.

Where methods used differed from those planned, recorded in the protocol and in the PROSPERO registration, they are described in Table 3.

Table 3. Differences in review methodology to protocol plan

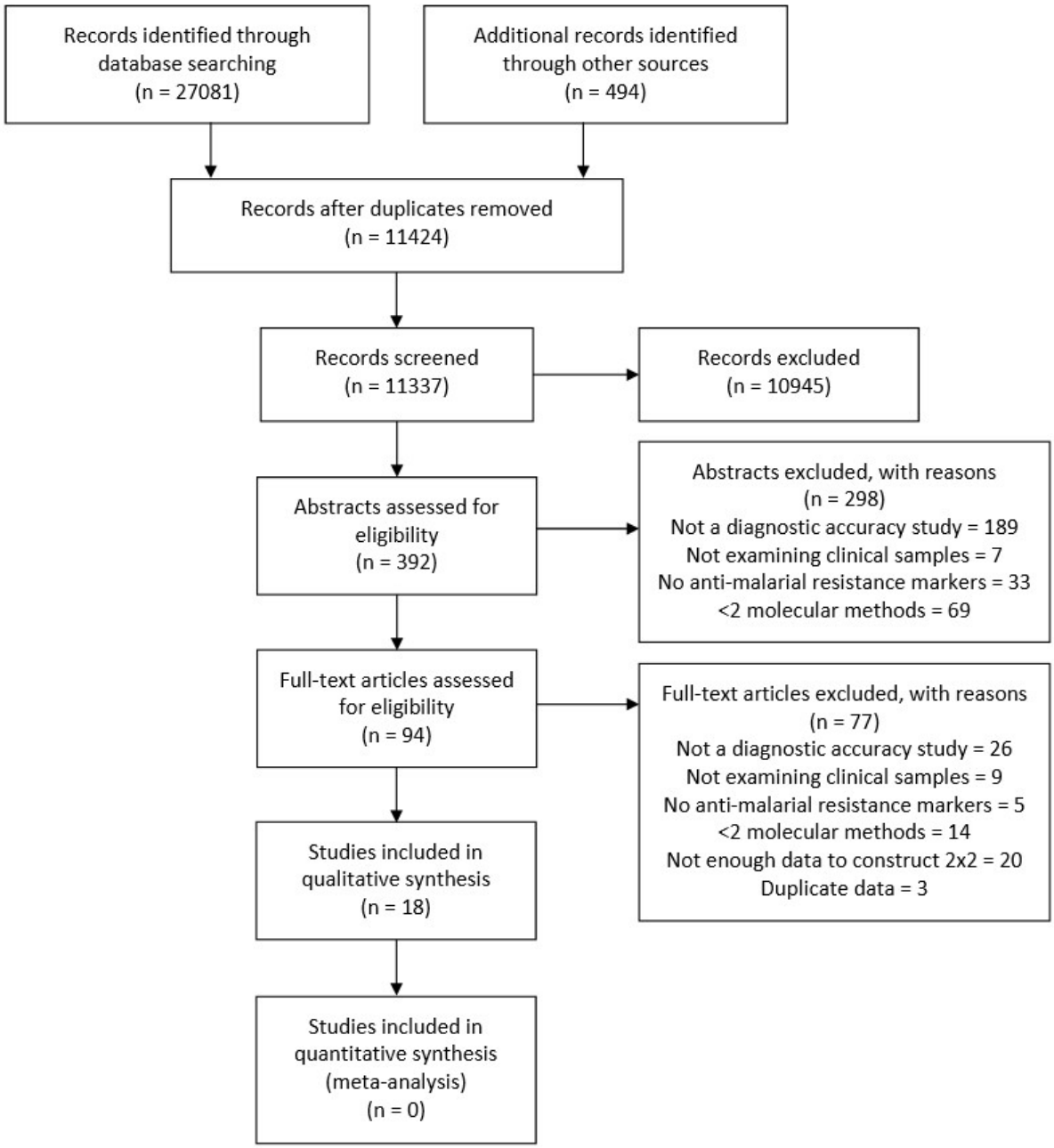
Protocol	**Review**
"Where studies examine patients or samples in or from areas where *Plasmodium falciparum* is responsible for <90% of confirmed cases, only studies using a PCR-based method of speciation will be included."	Studies examining patient or samples from all areas were included, regardless of species prevalence or determination.
"A second reviewer will independently screen 10% of search results and all reports deemed eligible by the first reviewer. We will resolve disagreements through discussion."	Only one reviewer screened 100% of search results.
"A second reviewer independently extract data. Discrepancies will be resolved by discussion."	Only one reviewer extracted data.
"We will assess risk of bias using the piloted adapted QUADAS-2 tool... For each domain two reviewers will independently make an assessment of the risk of bias from the extracted information... Disagreements will be resolved by discussion."	Only one reviewer made an assessment of the risk of bias and applicability concerns.
“Data were extracted…using the data extraction form.”	Data were extracted using the data extraction form and results data were extracted directly into 3x3 tables for test accuracy results, in Figure 3.
“We will assess the quality of evidence using Grading of Recommendations Assessment, Development and Evaluation (GRADE) methodology”	GRADE could not be applied in a way that met the required criteria, and would not be a useful way of assessing the evidence.

Results

Search results

Searches of MEDLINE, EMBASE, BOISIS and Science Citation Index returned a total of 27,081 results; searches of reference lists of eligible studies returned another 494 hits. Emails to authors of eligible studies and other experts in the field did not produce any suggestions of studies that may be eligible. Of these 27,575 results, 11,424 were identified as unique reports. 10,945 of these were excluded based on screening of the title, another 298 were excluded based on screening of the abstract. The full-text of 94 papers was examined; of these 15 were initially eligible. Results were successfully sought for another three studies of the 22 potentially eligible studies, leaving 77 ineligible and 18 eligible. One report is counted as both eligible and ineligible as data were available for one comparison and not for a second. Figure 5 shows a flow chart of the movement of studies through this review.

Figure 5: PRISMA flow diagram



Eligible studies

18 studies were deemed to be eligible; listed in Table 4.

Table 4. Eligible studies

Study	Title
Durand et al. (2000)	Use of molecular beacons to detect an antifolate resistance-associated mutation in *Plasmodium falciparum*
Durand et al. (2002)	Rapid detection of a molecular marker for chloroquine-resistant *falciparum* malaria
Kengne et al. (2003)	Malaria: use of restriction endonuclease digestion and mutation-specific PCR for antifolate resistance isolate detection
Alker et al. (2004)	Rapid real-time PCR genotyping of mutations associated with sulfadoxine-pyrimethamine resistance in *Plasmodium falciparum*
Wilson et al. (2005b)	Prevalence of *pfcrt* mutations in Congolese and Malawian *Plasmodium falciparum* isolates as determined by a new Taqman assay
Farcas et al. (2006)	Real-time polymerase chain reaction assay for the rapid detection and characterization of chloroquine-resistant *Plasmodium falciparum* malaria in returned travellers
Juliano et al. (2007)	Minority-variant *pfcrt* K76T mutations and chloroquine resistance, Malawi
Juliano et al. (2008)	Detection of the *dihydrofolate reductase*-164L mutation in *Plasmodium falciparum* infections from Malawi by heteroduplex tracking assay
Mens et al. (2008)	Real-time PCR/MCA assay using fluorescence resonance energy transfer for the genotyping of resistance related *dhps*-540 mutations in *Plasmodium falciparum*
Andriantsoanirina et al. (2009)	Rapid detection of point mutations in *Plasmodium falciparum* genes associated with antimalarial drugs resistance by using High-Resolution Melting analysis
Juliano et al. (2009)	Nonradioactive heteroduplex tracking assay for the detection of minority-variant chloroquine-resistant *Plasmodium falciparum* in Madagascar
Cruz et al. (2010)	High-throughput genotyping of single nucleotide polymorphisms in the *Plasmodium falciparum dhfr* gene by asymmetric PCR and melt-curve analysis
Gan and Loh (2010)	Rapid identification of chloroquine and atovaquone drug resistance in *Plasmodium falciparum* using high-resolution melt polymerase chain reaction
Muhamad et al. (2011)	SYBR Green I and TaqMan quantitative real-time polymerase chain reaction methods for the determination of amplification of *Plasmodium falciparum multidrug resistance-1 gene (pfmdr1)*
Moers et al. (2015)	Detection of single-nucleotide polymorphisms in *Plasmodium falciparum* by PCR primer extension and lateral flow immunoassay
Sharma et al. (2016)	Disagreement in genotyping results of drug resistance alleles of the *Plasmodium falciparum dihydrofolate reductase* (*Pfdhfr*) gene by allele-specific PCR (ASPCR) assays and Sanger sequencing
Chahar et al. (2017)	Establishment and application of a novel isothermal amplification assay for rapid detection of chloroquine resistance (K76T) in *Plasmodium falciparum*
Pholwat et al. (2017)	The Malaria TaqMan Array Card: 87 assays for *Plasmodium falciparum* drug resistance, speciation, and genotyping in a single reaction

Antimalarial markers

All but one of the antimalarial markers selected for this review, *pfdhfr* A16V, are included in at least one eligible study. The majority of markers are included in one or two studies, while some markers, particularly those in the genes *pfcrt* and *pfdhfr,* are included in five to ten studies. Table 5 lists which genotypes and haplotypes were included in each of the eligible studies. Eligible studies also include data on antimalarial markers that were not selected for this review, such as other markers in *pfk13*.

Table 5. Molecular markers, by study

		Study																	
Gene	**Marker**	Durand et al. (2000)	Durand et al. (2002)	Kengne et al. (2003)	Alker et al. (2004)	Wilson et al. (2005b)	Farcas et al. (2006)	Juliano et al. (2007)	Juliano et al. (2008)	Mens et al. (2008)	Andriantsoanirina et al. (2009)	Juliano et al. (2009)	Cruz et al. (2010)	Gan and Loh (2010)	Muhamad et al. (2011)	Moers et al. (2015)	Sharma et al. (2016)	Chahar et al. (2017)	Pholwat et al. (2017)
pfcrt	76		✓			✓	✓				✓	✓				✓		✓	
	72-76							✓						✓					✓
pfcytb	268													✓					✓
pfdhfr	51				✓						✓		✓				✓		✓
	59				✓						✓		✓				✓		✓
	108	✓		✓	✓						✓		✓				✓		✓
	164								✓		✓		✓				✓		✓
pfdhps	436				✓						✓								
	437				✓						✓								
	540				✓					✓	✓								✓
	581				✓														✓
	613				✓														✓
	436-437																		✓
pfk13	458																		✓
	493																		✓
	539																		✓
	543																		✓
	561																		✓
	580																		✓
pfmdr1	86										✓					✓			✓
	184															✓			✓
	1034																		✓
	1042																		✓
	1246															✓			✓
	copy number														✓				

Molecular methods of detection

Table 6 lists the molecular methods included in each study. When different studies used methods of the same name, often there was variation in the exact method, for example, in PCR cycling conditions or gel visualisation. The index test methods have also been grouped more broadly by the technique used for DNA amplification and detection - for example, all methods requiring RT-PCR machines are grouped together.

Table 6. Molecular methods, by study

	Study																	
Method[1]	Durand et al. (2000)	Durand et al. (2002)	Kengne et al. (2003)	Alker et al. (2004)	Wilson et al. (2005b)	Farcas et al. (2006)	Juliano et al. (2007)	Juliano et al. (2008)	Mens et al. (2008)	Andriantsoanirina et al. (2009)	Juliano et al. (2009)	Cruz et al. (2010)	Gan and Loh (2010)	Muhamad et al. (2011)	Moers et al. (2015)	Sharma et al. (2016)	Chahar et al. (2017)	Pholwat et al. (2017)
PCR-RFLP	**R**	**R**	**R**			**R**			**R**		**R**		**R**					
ASPCR			**I**		**R**		**R**									**I**		
PCR with molecular beacons	**I**	**I**																
PCR-MSS-HTA							**I**	**I**			**R**							
PCR-DIG-HTA											**I**							
PCR-HRM													**I**					
PCR-PEXT-NALFIA															**I**			
RT-PCR				**I**	**I**	**I**		**R**				**I**	**R**	**R**	**I**	**R**		
RT-PCR-MCA												**I**						
RT-PCR-HRM										**I**								
RT-PCR-FRET/MCA									**I**									
LAMP (visible)																	**I**	
LAMP (gel)																	**I**	
TAC																		**I**
Sequencing				**R**						**R**		**R**			**R**	**R**	**R**	**R**

[1] R = Reference test, I = Index test

Characteristics of studies

Table 31 to Table 48 detail the main characteristics of all 18 eligible studies. Often it was not possible to extract the information required to complete the data extraction form defined in the protocol for this review. Sometimes data could be inferred where it was not stated explicitly, for example, the type of laboratory carrying out the molecular analysis.

Study Design and Setting

Sampling Method

Only one report describes the sampling method of the study, likely to be the only study to have used consecutive recruitment (Farcas et al., 2006). For all other studies, sampling is not clearly described, studies may have had a case-control design where samples were chosen based on previous determination of genotype, or known characteristics such as parasitaemia.

Laboratory

The type of laboratory where tests were performed was inferred where possible; none are explicitly reported. They include hospital diagnostic labs, parasitology reference labs, university research labs, and national defence research labs.

Dates

The dates during which the studies were carried out are not described; a wide and inclusive range could be deduced for some studies from the date of patient screening or diagnosis, and the date of the publication of the results. Ten studies describe the dates that patients were screened or diagnosed, or the study dates for larger studies from which samples were selected (Durand et al., 2000, Alker et al., 2004, Andriantsoanirina et al., 2009, Pholwat et al., 2017, Mens et al., 2008, Durand et al., 2002, Wilson et al., 2005b, Farcas et al., 2006, Juliano et al., 2009, Moers et al., 2015). The dates that patients were likely infected are not described by any report.

Location

Five studies were carried out in non-endemic North America and Europe using samples taken from travellers returning from malarious areas (Cruz et al., 2010, Moers et al., 2015, Farcas et al., 2006, Durand et al., 2002, Durand et al., 2000). The locations of infection were described as; “Africa”, “mostly African countries”, “Africa, the Indian subcontinent, South and Central America, Oceania and Southeast Asia”, and “world-wide”. 13 studies were carried out using samples from patients resident in malarious areas; only three of these studies were carried out in a malaria-endemic country (Sharma et al., 2016, Muhamad et al., 2011, Chahar et al., 2017). One study was carried out in non-endemic Singapore, but the origin of the samples is not described (Gan and Loh, 2010). Samples are described as being from Malawi (six), Madagascar (three), Malawi (three), Democratic Republic of Congo (two), India (two), Thailand (two), Burkina Faso (one), China (one), the Comoros Islands (one), Kenya (one), and Uganda (one).

Patients

Any details of patients, even ones highly relevant to test accuracy such as method of diagnosis, eligibility criteria, concomitant conditions, and number of patients, are described in only a minority of reports.

Eligibility criteria

Only one paper reports detailed eligibility criteria (Farcas et al., 2006). Symptomatic patients made up at least some of the participants in seven studies (Durand et al., 2000, Andriantsoanirina et al., 2009, Muhamad et al., 2011, Cruz et al., 2010, Durand et al., 2002, Farcas et al., 2006, Moers et al., 2015). Populations of five studies included some patients from screening programs who are likely to have been asymptomatic (Alker et al., 2004, Juliano et al., 2008, Wilson et al., 2005b, Juliano et al., 2007, Juliano et al., 2009). All other reports do not describe this characteristic for any participants.

Diagnosis of malaria

Nine papers report the method of diagnosis of malaria for at least some participants: microscopy of blood smears (Alker et al., 2004, Juliano et al., 2008, Andriantsoanirina et al., 2009, Juliano et al., 2007, Juliano et al., 2009), RT-PCR (Cruz et al., 2010), microscopy and PCR (Sharma et al., 2016, Farcas et al., 2006, Moers et al., 2015), and histopathological examination of placental tissue (Alker et al., 2004, Juliano et al., 2008, Juliano et al., 2007). All other studies do not describe the method of diagnosis.

Sex, age, and concomitant conditions

Six studies report the sex of at least some of the patients from whom samples were taken. Five studies included women (Alker et al., 2004, Juliano et al., 2008, Wilson et al., 2005b, Juliano et al., 2007, Juliano et al., 2009), one study had a ratio of 5:8 women: men (Farcas et al., 2006). Age is described for four studies; adults and children in three (Farcas et al., 2006, Juliano et al., 2009, Wilson et al., 2005b), adults in one (Juliano et al., 2007), and two additional studies included pregnant women, likely, the majority of who were adults (Alker et al., 2004, Juliano et al., 2008). Two study reports describe HIV status and one of these also describes haemoglobin status (Juliano et al., 2007, Alker et al., 2004).

Number of participants

Four papers describe the number of patients from whom the samples for testing had been taken; the number of participants ranges from 24-200 (Alker et al., 2004, Juliano et al., 2008, Farcas et al., 2006, Juliano et al., 2007).

Samples

Source of samples

The source of blood for testing is described briefly for 11 studies: peripheral (Alker et al., 2004, Juliano et al., 2008, Juliano et al., 2007, Juliano et al., 2009, Moers et al., 2015), finger prick (Mens et al., 2008, Sharma et al., 2016), venous (Durand et al., 2000, Alker et al., 2004, Andriantsoanirina et al., 2009, Durand et al., 2002), and with EDTA as an anticoagulant (Andriantsoanirina et al., 2009, Wilson et al., 2005b).

Parasitaemia

Nine papers give some description of parasitaemia for at least some of the samples included: two upper bounds, two lower bounds, three averages and seven ranges (Mens et al., 2008, Cruz et al., 2010, Durand et al., 2002, Wilson et al., 2005b, Farcas et al., 2006, Juliano et al., 2007, Juliano et al., 2009, Gan and Loh, 2010, Moers et al., 2015). Authors of one paper shared unpublished individual parasitaemias (Cruz et al., 2010).

Speciation

Six papers describe speciation by PCR (Sharma et al., 2016, Farcas et al., 2006, Gan and Loh, 2010), RT-PCR (Cruz et al., 2010), and microscopy (Alker et al., 2004, Andriantsoanirina et al., 2009, Farcas et al., 2006).

Multiplicity of infection

No reports describe multiplicity of infection.

Sample storage

Eleven papers describe some aspect of sample storage conditions: filter paper (Juliano et al., 2008, Muhamad et al., 2011, Sharma et al., 2016, Juliano et al., 2007, Juliano et al., 2009), filter paper at room temperature in an air-tight bag (Mens et al., 2008), Isocode Stix, desiccated (Wilson et al., 2005b), purified DNA (Pholwat et al., 2017), dried blood spots (Pholwat et al., 2017), blood pellet (Pholwat et al., 2017), pellets at -20°C (Kengne et al., 2003), +4°C within 24 hours of collection (Andriantsoanirina et al., 2009), frozen blood (Juliano et al., 2008), plasma and packed-cell fractions stored at -80°C, thawed and drops shipped on filter paper (Alker et al., 2004), -80°C (Wilson et al., 2005b).

Number of samples

The number of samples ranged from 24-200. One study included multiple samples from the same participants (Juliano et al., 2009). It is possible but unlikely that studies examined the same samples:

- Durand et al. (2000) and Durand et al. (2002) do not use the same samples but Kengne et al. (2003) may overlap with either or both of them
- Cruz et al. (2010) and Farcas et al. (2006)
- Sharma et al. (2016) and Chahar et al. (2017)
- Muhamad et al. (2011) and Pholwat et al. (2017)
- Gan and Loh (2010) and any other study
- Andriantsoanirina et al. (2009) and Juliano et al. (2009)
- Alker et al. (2004) and Wilson et al. (2005b) do not use the same samples but Juliano et al. (2008), Juliano et al. (2007) and Juliano et al. (2009) may overlap with either or both of them

It is likely that there was at least some of the same samples were used in Juliano et al. (2008), Juliano et al. (2007) and Juliano et al. (2009).

DNA extraction

Each of the studies used the same method of DNA extraction for samples tested by index and reference tests. Qiagen reagents using minicolumn purification were used in 11 studies (Alker et al., 2004, Juliano et al., 2008, Pholwat et al., 2017, Muhamad et al., 2011, Sharma et al., 2016, Wilson et al., 2005b, Farcas et al., 2006, Juliano et al., 2009, Gan and Loh, 2010, Moers et al., 2015, Chahar et al., 2017), two studies used ethanol precipitation (Durand et al., 2000, Durand et al., 2002), one chelex extraction (Kengne et al., 2003), one phenol-chloroform (Andriantsoanirina et al., 2009), one Isocode Stix elution (Wilson et al., 2005b), and one magnetic beads (Cruz et al., 2010). Two reports do not describe DNA extraction methods (Mens et al., 2008, Juliano et al., 2007).

Index test and reference test

Index tests

13 different index tests were examined, nine of which were only studied once. Methods used exclusively as index tests were:

- Loop-Mediated Isothermal Amplification (LAMP) (Chahar et al., 2017)
- PCR with molecular beacons (Durand et al., 2000, Durand et al., 2002)
- Polymerase Chain Reaction Digoxigenin-labeled Chemiluminescent Heteroduplex Tracking Assay (PCR-DIG-HTA) (Juliano et al., 2009)
- Polymerase Chain Reaction with High-Resolution Melt (PCR-HRM) (Gan and Loh, 2010)
- Polymerase Chain Reaction with Primer Extension and Lateral Flow Immunoassay (PCR-PEXT-NALFIA) (Moers et al., 2015)
- Real-Time Polymerase Chain Reaction with Melting Curve Analysis (RT-PCR-MCA) (Cruz et al., 2010)
- High-Resolution Melt Real-Time Polymerase Chain Reaction (RT-PCR-HRM) (Andriantsoanirina et al., 2009)

- Real-Time Polymerase Chain Reaction and Melting Curve Analysis using Fluorescence resonance energy transfer (RT-PCR-FRET/MCA) (Mens et al., 2008)
- TaqMan Array Card (Pholwat et al., 2017).

Used as both index and reference tests were:

- Allele-Specific Polymerase Chain Reaction (ASPCR) (Kengne et al., 2003, Sharma et al., 2016, Wilson et al., 2005b, Juliano et al., 2007)
- Polymerase Chain Reaction with Multiple Site Specific Heteroduplex Tracking Assay (PCR-MSS-HTA) (Juliano et al., 2008, Juliano et al., 2007)
- Real-Time Polymerase Chain Reaction (RT-PCR) (Juliano et al., 2008, Alker et al., 2004, Andriantsoanirina et al., 2009, Muhamad et al., 2011, Cruz et al., 2010, Wilson et al., 2005b, Farcas et al., 2006, Gan and Loh, 2010, Moers et al., 2015).

Reference test

Used exclusively as reference tests, each in seven studies, were:

- Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) (Durand et al., 2000, Kengne et al., 2003, Mens et al., 2008, Durand et al., 2002, Farcas et al., 2006, Juliano et al., 2009, Gan and Loh, 2010)
- Sequencing (Alker et al., 2004, Andriantsoanirina et al., 2009, Pholwat et al., 2017, Cruz et al., 2010, Sharma et al., 2016, Moers et al., 2015, Chahar et al., 2017)

  Only two papers compared the same index (RT-PCR) and reference test (sequencing) for the same molecular markers - Alker et al. (2004) and Cruz et al. (2010) for *pfdhfr* 51, 59 and 108.

Interpretation

For index and reference tests, some description of the method by which genotype was interpreted is given 12 reports (Durand et al., 2000, Alker et al., 2004, Juliano et al., 2008, Andriantsoanirina et al., 2009, Cruz et al., 2010, Sharma et al., 2016, Durand et al., 2002, Wilson et al., 2005b, Farcas et al., 2006, Juliano et al., 2007, Gan and Loh, 2010, Chahar et al., 2017).

Funding

Funding from industry contributed to four studies (Durand et al., 2000, Farcas et al., 2006, Juliano et al., 2008, Juliano et al., 2009), and from other sources to 15 studies (Durand et al., 2000, Kengne et al., 2003, Alker et al., 2004, Wilson et al., 2005b, Farcas et al., 2006, Juliano et al., 2007, Juliano et al., 2008, Mens et al., 2008, Andriantsoanirina et al., 2009, Juliano et al., 2009, Muhamad et al., 2011, Sharma et al., 2016, Chahar et al., 2017, Moers et al., 2015, Pholwat et al., 2017). Funding sources were not described for three studies (Cruz et al., 2010, Durand et al., 2002, Gan and Loh, 2010).

Methodological quality of included studies

Figure 6 shows the overall risk of bias and applicability concerns for all studies. Figure 7 shows the risk of bias and applicability concerns for individual studies. Table 49 to Table 66 record the assessments for each individual study.

Figure 6: Overall risk of bias and applicability concerns

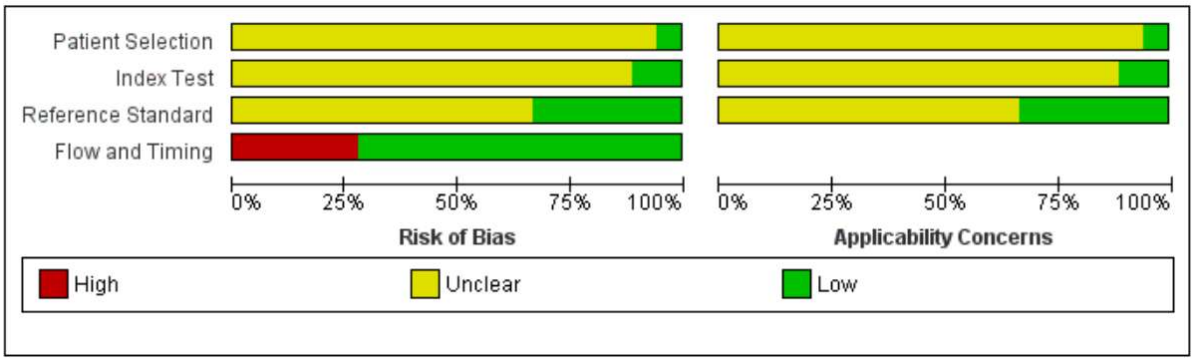
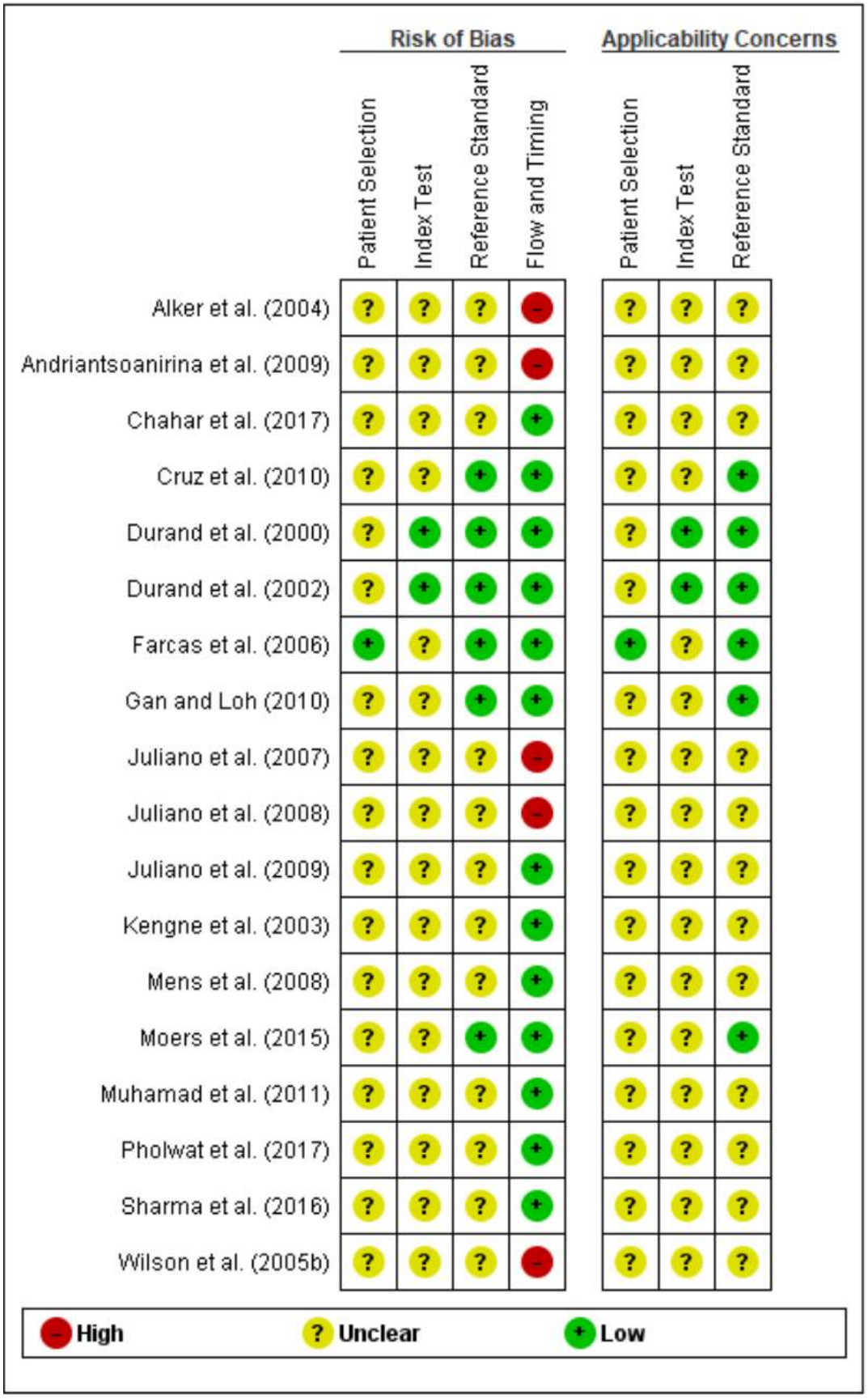


Figure 7: Risk of bias and applicability concerns for each study



Patient Selection

In the patient selection domain, one study was at low risk of bias as consecutive samples of patients and samples were enrolled, a case-control design was avoided, samples had a range of parasitaemias, and inappropriate exclusions were avoided (Farcas et al., 2006). All other studies were all at an unclear risk of bias as insufficient information on patient and sample selection is available.

One study, Farcas et al. (2006), was of low concern regarding applicability because included patients closely matched the review question. The remaining studies were all of unclear applicability as insufficient information on patient and sample selection is available.

Index Test

In the index test domain, two studies were at low risk of bias as index test results were interpreted without knowledge of the results of the reference standard, and thresholds used were pre-specified and reasonable (Durand et al., 2000, Durand et al., 2002). The remaining studies were all at an unclear risk of bias as insufficient information on index test conduct is available.

Two studies were of low concern regarding applicability the index test conduct and interpretation were carried out in the way they would be in a surveillance program. (Durand et al., 2000, Durand et al., 2002). The remaining studies were all of unclear applicability as insufficient information on index test conduct is available.

Reference Test

In the reference test domain, five studies were at low risk of bias as the reference standard is likely to correctly classify the target condition and results were interpreted without knowledge of the index test results (Cruz et al., 2010, Durand et al., 2002, Farcas et al., 2006, Gan and Loh, 2010, Moers et al., 2015). One study was at low risk of bias as the reference standard is likely to correctly classify the target condition, results were interpreted without knowledge of the index test results, and thresholds

used were pre-specified and reasonable (Durand et al., 2000). The remaining studies were all at an unclear risk of bias as insufficient information on reference test conduct is available.

Six studies were of low concern regarding applicability (Durand et al., 2000, Durand et al., 2002, Farcas et al., 2006, Gan and Loh, 2010, Moers et al., 2015, Cruz et al., 2010) because the reference standard defines the target condition in a way that matches the review question. The remaining studies were all of unclear applicability as insufficient information on reference test conduct is available.

Flow and Timing

In the flow and timing domain, two studies were at a low risk of bias although the interval between index and reference test was not described, as it is very likely that the same patient sample was used for testing, and although not all patients received the same reference standard, all patients were included in the analysis (Pholwat et al., 2017, Muhamad et al., 2011). 11 studies were at low risk of bias although the interval between index and reference test was not described, as it is very likely that the same patient sample was used for testing (Durand et al., 2000, Kengne et al., 2003, Mens et al., 2008, Cruz et al., 2010, Sharma et al., 2016, Durand et al., 2002, Farcas et al., 2006, Juliano et al., 2009, Gan and Loh, 2010, Moers et al., 2015, Chahar et al., 2017). Five studies are at high risk of bias as not all samples were included in the analysis (Juliano et al., 2008, Andriantsoanirina et al., 2009, Wilson et al., 2005b), or, as not all samples received the reference test, and not all samples were included in the analysis (Alker et al., 2004, Juliano et al., 2007).

Sensitivity and Specificity

Plasmodium falciparum chloroquine resistance transporter

pfcrt 76

Seven studies using six different index tests (one index test with two different methods of interpretation) and five different reference tests examined *pfcrt*76; Durand et al. (2002), Wilson et al. (2005b), Farcas et al. (2006), Andriantsoanirina et al. (2009), Juliano et al. (2009), Moers et al. (2015) and Chahar et al. (2017). Estimates of sensitivity range from 72.7% (57.2-85.0) using PCR-PEXT-NALFIA to 100% (95.5-100.0) using PCR with molecular beacons. Specificity estimates range from 83.3% (35.9-99.6) to 100% (92.9-100.0) with PCR-PEXT-NALFIA and PCR with molecular beacons. Six of the seven methods could be up to 100% sensitive and specific; PCR with molecular beacons, PCR-DIG-HTA, RT-PCR, RT-PCR-HRM and LAMP (visible and gel interpretation).

Table 7. Sensitivity and specificity for pfcrt 76

	pfcrt 76[2]	reference test				
		PCR-RFLP	**ASPCR**	**PCR-MSS-HTA**	**RT-PCR**	**Sequencing**
index test	**PCR with molecular beacons**	Durand et al. (2000) 100% (95.5-100.0) Durand et al. (2000) 100% (92.9-100.0)				
	PCR-DIG-HTA	- Juliano et al. (2009)[4] 93.3% (77.9-99.2)	-	Juliano et al. (2009)[3] 87.5% (47.3-99.7) Juliano et al. (2009)[3] 100% (78.2-100.0) Juliano et al. (2009)[4] 100% (15.8-100.0) Juliano et al. (2009)[4] 100% (87.7-100.0)		
	PCR-PEXT-NALFIA				Moers et al. (2015)[5] 81.8% (48.2-97.7) Moers et al. (2015)[5] 83.3% (35.9-99.6) Moers et al. (2015)[6] 72.7% (57.2-85.0) Moers et al. (2015)[6] 85.0% (70.2-94.3)	

[2] Shaded light grey = sensitivity, shaded dark grey = specificity
[3] Samples from Malawi
[4] Samples from Madagascar
[5] Samples from Kenya
[6] Samples from the Malaria Reference Laboratory

	RT-PCR	Farcas et al. (2006) 100% (95.3-100.0)	Wilson et al. (2005b) 100% (93.2-100.0)			
		Farcas et al. (2006) 94.1% (83.8-98.8)	Wilson et al. (2005b) 100% (73.5-100.0)			
	RT-PCR-HRM					Andriantsoanirina et al. (2009) 100% (83.2-100.0)
						Andriantsoanirina et al. (2009) 100% (90.1-100.0)
	LAMP (visible)					Chahar et al. (2017) 100% (76.8-100.0)
						Chahar et al. (2017) 100% (90.3-100.0)
	LAMP (gel)					Chahar et al. (2017) 100% (76.8-100.0)
						Chahar et al. (2017) 100% (90.3-100.0)

pfcrt 72-76

Three studies using three different index tests and three different reference tests examined *pfcrt*72-76; Juliano et al. (2007), Gan and Loh (2010) and Pholwat et al. (2017). Sensitivity estimates are both 100%, with CI between 87.7-100.0 (TAC) and 92.6-100.0 (RT-PCR-HRM). Specificity estimates range from 48.8% (32.9-64.9) to 100% (76.8-100.0) using PCR-DIG-HTA and TAC. Two methods could be up to 100% sensitive and specific; RT-PCT-HRM and TAC.

Table 8. Sensitivity and specificity for pfcrt 72-76

***pfcrt* 72-76**[2]		**reference test**		
		ASPCR	**RT-PCR**	**Sequencing**
index test	**PCR-MSS-HTA**	-		
		Juliano et al. (2007)[7] 96.3% (81.0-99.9)		
		-		
		Juliano et al. (2007)[8] 48.8% (32.9-64.9)		
	RT-PCR-HRM		Gan and Loh (2010) 100% (92.6-100.0)	
			Gan and Loh (2010) 100% (47.8-100.0)	
	TAC			Pholwat et al. (2017) 100% (87.7-100.0)
				Pholwat et al. (2017) 100% (76.8-100.0)

[7] Samples from Blantyre, Malawi

[8] Samples from Mpemba and Madziabango, Malawi

Plasmodium falciparum cytochrome b

***pfcytb* 268**

Two studies using two different index tests and two different reference tests examined *cytochrome b* (*pfcytb*) 268; Gan and Loh (2010) and Pholwat et al. (2017). No estimate of sensitivity could be made as no resistant genotypes were found. The two estimates of specificity are both 100% with CI of 91.6-100.0 (TAC) and 93.3-100.0 (RT-PCR-HRM).

Table 9. Sensitivity and specificity for pfcytb

pfcytb 268[2]		**reference test**	
		PCR-RFLP	**sequencing**
index test	**RT-PCR-HRM**	- Gan and Loh (2010) 100% (93.3-100.0)	
	TAC		- Pholwat et al. (2017) 100% (91.6-100.0)

Plasmodium falciparum dihydrofolate reductase

pfdhfr 51

Five studies using five different index tests and one reference test examined *pfdhfr* 51; Alker et al. (2004), Andriantsoanirina et al. (2009), Cruz et al. (2010), Sharma et al. (2016) and Pholwat et al. (2017). Estimates of sensitivity range from 78.8% (61.1-91.1) using RT-PCR to 100% (90.7-100.0) using TAC. Specificity estimates range from 71.4% (56.7-83.4) to 100% (39.8-100.0) using ASPCR and TAC. TAC and RT-PCR-HRM are the methods that combine the highest sensitivity and specificities.

Table 10. Sensitivity and specificity for pfdhfr 51

	***pfdhfr* 51[2]**	**reference test**
		sequencing
index test	**ASPCR**	Sharma et al. (2016) 100% (54.1-100.0)
		Sharma et al. (2016) 71.4% (56.7-83.4)
	RT-PCR	Alker et al. (2004) 100% (47.8-100.0)
		-
		Cruz et al. (2010) 78.8% (61.1-91.1)
		Cruz et al. (2010) 81.1% (48.2-97.7)
	RT-PCR-MCA	Cruz et al. (2010) 87.9% (71.8-96.6)
		Cruz et al. (2010) 90.1% (58.7-99.8)
	RT-PCR-HRM	Andriantsoanirina et al. (2009) 100.0% (87.7-100.0)
		Andriantsoanirina et al. (2009) 90.3% (74.2-96.0)
	TAC	Pholwat et al. (2017) 100% (90.7-100.0)
		Pholwat et al. (2017) 100% (39.8-100.0)

pfdhfr 59

Five studies using five different index tests and one reference test examined *pfdhfr* 59; Alker et al. (2004), Andriantsoanirina et al. (2009), Cruz et al. (2010), Sharma et al. (2016) and Pholwat et al. (2017). Estimates of sensitivity range from 71.7% (57.7-83.2) by Sharma et al. (2016) to 100% (91.4-100.0) by Pholwat et al. (2017). Specificity estimates range from 70.0% (34.8-93.3) to 100% (69.2-100.0), both by Cruz et al. (2010) with different methods. Overall, it appears as though TAC or RT-PCR-HRM may be the most sensitive methods, while either of these, or RT-PCR might be the most specific.

Table 11. Sensitivity and specificity for pfdhfr 59

***pfdhfr* 59[2]**		**reference test**
		sequencing
index test	**ASPCR**	Sharma et al. (2016) 71.7% (57.7-83.2)
		Sharma et al. (2016) 100% (15.8-100.0)
	RT-PCR	Alker et al. (2004) 100% (39.8-100.0)
		Alker et al. (2004) 100% (2.5-100.0)
		Cruz et al. (2010) 85.3% (68.9-95.0)
		Cruz et al. (2010) 100% (69.2-100.0)
	RT-PCR-MCA	Cruz et al. (2010) 73.5% (55.6-87.1)
		Cruz et al. (2010) 70.0% (34.8-93.3)
	RT-PCR-HRM	Andriantsoanirina et al. (2009) 100% (87.7-100.0)
		Andriantsoanirina et al. (2009) 90.3% (74.2-98.0)
	TAC	Pholwat et al. (2017) 100% (91.4-100.0)
		Pholwat et al. (2017) 100% (2.5-100.0)

pfdhfr 108

Seven studies using six different index tests and two different reference tests examined *pfdhfr* 108; Alker et al. (2004), Andriantsoanirina et al. (2009), Cruz et al. (2010), Sharma et al. (2016), Durand et al. (2000), Kengne et al. (2003) and Pholwat et al. (2017). The least sensitive method appears to be RT-PCR 27.0% (0.0-14.2), the most RT-PCR-HRM 100% (94.4-100.0). Specificity ranges from 0.0% (0.0-41.0) with RT-PCR to 100.0% (91.4-100.0).

Table 12. Sensitivity and specificity for pfdhfr 108

***pfdhfr* 108[2]**		**reference test**	
		PCR-RFLP	**sequencing**
index test	**ASPCR**	Kengne et al. (2003) 100% (93.6-100.0)	Sharma et al. (2016) 100% (91.0-100.0)
		-	Sharma et al. (2016) 81.3% (54.4-96.0)
	PCR with molecular beacons	Durand et al. (2000) 100% (93.9-100.0)	
		Durand et al. (2000) 100% (91.4-100.0)	
	RT-PCR		Alker et al. (2004) 100% (47.8-100.0)
			-
			Cruz et al. (2010) 27.0% (0.0-14.2)
			Cruz et al. (2010) 0.0% (0.0-41.0)
	RT-PCR-MCA		Cruz et al. (2010) 81.1% (64.8-92.0)
			Cruz et al. (2010) 71.4% (29.0-96.3)
	RT-PCR-HRM		Andriantsoanirina et al. (2009) 100% (94.4-100.0)
			Andriantsoanirina et al. (2009) 98.0% (89.6-100.0)
	TAC		Pholwat et al. (2017) 100% (91.6-100.0)
			-

pfdhfr 164

Five studies using six different index tests and two different reference tests examined *pfdhfr* 164; Juliano et al. (2008), Andriantsoanirina et al. (2009), Cruz et al. (2010), Sharma et al. (2016) and Pholwat et al. (2017). All tests for which sensitivity could be calculated reach 100%, but the confidence intervals are wide, from 39.8-100.0 for both ASPCR and PCR-MSS-HTA, to 85.8-100.0 for TAC. Specificity ranges from 7.8% (2.2-18.9) using ASPCR to 100% (92.1-100.0) using PCR-MSS-HTA. Both RT-PCR-HRM and TAC may achieve both 100% sensitivity and specificity.

Table 13. Sensitivity and specificity for pfdhfr 164

	***pfdhfr* 164[2]**	**reference test**	
		RT-PCR	**sequencing**
index test	**ASPCR**		Sharma et al. (2016) 100% (39.8-100.0)
			Sharma et al. (2016) 7.8% (2.2-18.9)
	PCR-MSS-HTA	Juliano et al. (2008)[7] 100% (39.8-100.0)	
		Juliano et al. (2008)[7] 90.1% (81.5-95.6)	
		-	
		Juliano et al. (2008)[8] 100% (92.1-100.0)	
	RT-PCR		-
			Cruz et al. (2010) 40% (25.7-55.7)
	RT-PCR-MCA		-
			Cruz et al. (2010) 82.2% (67.9-92.0)
	RT-PCR-HRM		Andriantsoanirina et al. (2009) 100% (47.8-100.0)
			Andriantsoanirina et al. (2009) 100% (85.8-100.0)
	TAC		Pholwat et al. (2017) 100% (85.8-100.0)
			Pholwat et al. (2017) 100% (81.5-100.0)

Plasmodium falciparum dihydropteroate synthase

pfdhps 436

Two studies using two different index tests and one reference test examined *pfdhps* 436; Alker et al. (2004) and Andriantsoanirina et al. (2009). Sensitivity could only be calculated for RT-PCR-HRM; 74.1% (53.7-89.9). Specificity is higher with RT-PCR at 100% (47.8-100.0) than RT-PCR-HRM at 90.3% (74.2-98.0).

Table 14. Sensitivity and specificity for pfdhps 436

	***pfdhps* 436[2]**	**reference test**
		sequencing
index test	**RT-PCR**	-
		Alker et al. (2004) 100% (47.8-100.0)
	RT-PCR-HRM	Andriantsoanirina et al. (2009) 74.1% (53.7-88.9)
		Andriantsoanirina et al. (2009) 90.3% (74.2-98.0)

pfdhps 437

Two studies using two different index tests and one reference test examined *pfdhps* 437; Alker et al. (2004) and Andriantsoanirina et al. (2009). RT-PCR-HRM is the least sensitive, 74.1% (53.7-88.9), and specific, 90.3% (74.2-98.0), RT-PCR the most sensitive 100% (29.2-100.0) and specific 100% (15.8-100.0).

Table 15. Sensitivity and specificity for pfdhps 437

***pfdhps* 437[2]**		**reference test**
		sequencing
index test	**RT-PCR**	Alker et al. (2004) 100% (29.2-100.0)
		Alker et al. (2004) 100% (15.8-100.0)
	RT-PCR-HRM	Andriantsoanirina et al. (2009) 74.1% (53.7-88.9)
		Andriantsoanirina et al. (2009) 90.3% (74.2-98.0)

***pfdhps* 436-437**

One study using one index test and one reference test examined *pfdhps* 436-437; Pholwat et al. (2017). Only sensitivity could be calculated, 100% (91.6-100.0).

Table 16. Sensitivity and specificity for pfdhps 436-437

***pfdhps* 436-437[2]**		**reference test**
		sequencing
index test	**TAC**	Pholwat et al. (2017) 100% (91.6-100.0)
		-

pfdhps 540

Four studies using four different index tests and two different reference tests examined *pfdhps* 540; Alker et al. (2004), Mens et al. (2008), Andriantsoanirina et al. (2009) and Pholwat et al. (2017). Sensitivity ranges from 97.3% (85.8-99.9) with RT-PCR-MCA/FRET to 100% (91.6-100.0) with TAC. Specificity ranges from 87.1% (70.2-96.4) to 100% (87.7-100.0) with RT-PCR-MCA/FRET and RT-PCR-HRM.

Table 17. Sensitivity and specificity for pfdhps 540

	***pfdhps* 540[2]**	**reference test**	
		PCR-RFLP	**sequencing**
index test	**RT-PCR**		Alker et al. (2004) 100% (39.8-100.0)
			Alker et al. (2004) 100% (2.5-100.0)
	RT-PCR-HRM		Andriantsoanirina et al. (2009) 100% (2.5-100.0)
			Andriantsoanirina et al. (2009) 100% (87.7-100.0)
	RT-PCR-MCA/FRET	Mens et al. (2008) 97.3% (85.8-99.9)	
		Mens et al. (2008) 87.1% (70.2-96.4)	
	TAC		Pholwat et al. (2017) 100% (91.6-100.0)
			-

pfdhps 581

Two studies using two different index tests and one reference test examine *pfdhps* 581; Alker et al. (2004) and Pholwat et al. (2017). Both tests achieve 100% sensitivity and specificity, RT-PCR with wider CI (sensitivity 2.5-100.0, specificity 39.8-100.0) and TAC with confidence intervals 87.7-100.0 (sensitivity) and 76.8-100.0 (specificity).

Table 18. Sensitivity and specificity for pfdhps 581

***pfdhps* 581[2]**		**reference test**
		sequencing
index test	**RT-PCR**	Alker et al. (2004) 100% (2.5-100.0)
		Alker et al. (2004) 100% (39.8-100.0)
	TAC	Pholwat et al. (2017) 100% (87.7-100.0)
		Pholwat et al. (2017) 100% (76.8-100.0)

pfdhps 613

Two studies using two different index tests and one reference test examined *pfdhps* 613; Alker et al. (2004) and Pholwat et al. (2017). Sensitivity cannot be calculated for either test. Both achieve 100% specificity, with the confidence intervals of 47.8-100.0 (RT-PCR) and 91.6-100.0 (TAC).

Table 19. Sensitivity and specificity for pfdhps 613

***pfdhps* 613[2]**		**reference test**
		sequencing
index test	**RT-PCR**	-
		Alker et al. (2004) 100% (47.8-100.0)
	TAC	-
		Pholwat et al. (2017) 100% (91.6-100.0)

Plasmodium falciparum kelch 13

***pfk13* 458, 493, 539, 543, 561, 580**

One study comparing one index test and one reference test examined *pfk13* 458, 493, 539, 543, 561, and 580; Pholwat et al. (2017). Sensitivity could only be calculated for *pfk13* 561 (100% (2.5-100.0) as no resistant genotypes were detected for any other markers. Estimates of specificity are almost identical for all markers, 100% (91.4-100.0) for *pfk13* 561, and 100% (91.6-100.0) for all other markers.

Table 20. Sensitivity and specificity for pfk13

		***pfk13*[2]**	**reference test**
			sequencing
index test	**TAC**	***pfk13* 458**	-
			Pholwat et al. (2017) 100% (91.6-100.0)
		***pfk13* 493**	-
			Pholwat et al. (2017) 100% (91.6-100.0)
		***pfk13* 539**	-
			Pholwat et al. (2017) 100% (91.6-100.0)
		***pfk13* 543**	-
			Pholwat et al. (2017) 100% (91.6-100.0)
		***pfk13* 561**	Pholwat et al. (2017) 100% (2.5-100.0)
			Pholwat et al. (2017) 100% (91.4-100.0)
		***pfk13* 580**	-
			Pholwat et al. (2017) 100% (91.6-100.0)

Plasmodium falciparum multi-drug resistance transporter 1

pfmdr1 86

Three studies using three different index test and one reference test examined *pfmdr1* 86; Andriantsoanirina et al. (2009), Moers et al. (2015) and Pholwat et al. (2017). Sensitivity ranges from 74.3% (56.7-87.5) to 100% (63.1-100.0), both with PCR-PEXT-NALFIA. PCR-PEXT-NALFIA is also the least specific test at 75% (34.9-96.8) while TAC is the most specific test at 100% (90.5-100.0).

Table 21. Sensitivity and specificity for pfmdr1 86

***pfmdr1* 86[2]**		**reference test**
		sequencing
index test	**RT-PCR-HRM**	Andriantsoanirina et al. (2009) 75% (58.8-87.3)
		Andriantsoanirina et al. (2009) 83.3% (58.6-96.4)
	PCR-PEXT-NALFIA	Moers et al. (2015)[5] 100% (63.1-100.0)
		Moers et al. (2015)[5] 75% (34.9-96.8)
		Moers et al. (2015)[6] 74.3% (56.7-87.5)
		Moers et al. (2015)[6] 86.5% (78.0-92.6)
	TAC	Pholwat et al. (2017) 100% (47.8-100.0)
		Pholwat et al. (2017) 100% (90.5-100.0)

pfmdr1 184

Two studies using two different index test and one reference test examined *pfmdr1* 184; Moers et al. (2015) and Pholwat et al. (2017). PCR-PEXT-NALFIA is the least sensitive at 67.4% (56.5-77.2) and the least specific 91.3% (79.2-97.6) while TAC is the most sensitive at 100% (82.4-100.0) and specific 100% (85.2-100.0).

Table 22. Sensitivity and specificity for pfmdr1 184

***pfmdr1* 184[2]**		**reference test**
		sequencing
index test	**PCR-PEXT-NALFIA**	Moers et al. (2015)[5] 88.9% (51.8-99.7)
		Moers et al. (2015)[5] 100% (59.0-100.0)
		Moers et al. (2015)[6] 67.4% (56.5-77.2)
		Moers et al. (2015)[6] 91.3% (79.2-97.6)
	TAC	Pholwat et al. (2017) 100% (82.4-100.0)
		Pholwat et al. (2017) 100% (85.2-100.0)

***pfmdr1* 1034, 1042**

One study using one index test and one reference test examined *pfmdr1* 1034 and 1042; Pholwat et al. (2017). Only specificity could be calculated for 1034; 100% (91.6-100.0). Sensitivity for 1042 is 100% (2.5-100.0) while specificity is 100% (91.4-100.0).

Table 23. Sensitivity and specificity for pfmdr1 1034 and 1042

***pfmdr1*[2]**		**reference test**	
		sequencing	
index test	**TAC**	***pfmdr1* 1034**	-
			Pholwat et al. (2017) 100% (91.6-100.0)
		***pfmdr1* 1042**	Pholwat et al. (2017) 100% (2.5-100.0)
			Pholwat et al. (2017) 100% (91.4-100.0)

pfmdr1 1246

Two studies using three different index tests and one reference test examined *pfmdr1* 1246; Moers et al. (2015) and Pholwat et al. (2017). Sensitivity ranges from 75% (34.9-96.8) with PCR-PEXT-NALFIA to 100% (39.8-100.0) with TAC. Specificity ranges from 83.2% (74.7-89.7) to 100% (90.7-100.0) with the same tests.

Table 24. Sensitivity and specificity for pfmdr1 1246

	***pfmdr1* 1246**[2]	**reference test**
		sequencing
index test	**PCR-PEXT-NALFIA**	Moers et al. (2015)[5] 83.3% (35.9-99.6)
		Moers et al. (2015)[5] 100% (63.0-100.0)
		Moers et al. (2015)[6] 75% (34.9-96.8)
		Moers et al. (2015)[6] 83.2% (74.7-89.7)
	TAC	Pholwat et al. (2017) 100% (39.8-100.0)
		Pholwat et al. (2017) 100% (90.7-100.0)

pfmdr1 copy number

One study using one index test and one reference test examined *pfmdr1* copy number; Muhamad et al. (2011). Sensitivity of RT-PCR is 86.7% (59.5-98.3) and specificity is 89.6% (77.3-96.5).

Table 25. Sensitivity and specificity for pfmdr1 copy number

	***pfmdr1* copy number**[2]	**reference test**
		RT-PCR
index test	**RT-PCR**	Muhamad et al. (2011) 86.7% (59.5-98.3)
		Muhamad et al. (2011) 89.6% (77.3-96.5)

Registration of Studies

None of the 38 studies were registered, either prospectively or retrospectively. A study of adherence to rapid diagnostic tests, associated with one study, was registered but did not contain any information useful for this review (Clinicaltrials.gov, 2009). None of the studies had published protocols.

None of the studies eligible for this review, or studies eligible subject to supply of additional results, were reported following the current gold-standard for reporting of diagnostic test accuracy studies; STARD 2015 (Bossuyt et al., 2015, Fontela et al., 2009, Ferrante di Ruffano et al., 2017). Reporting was generally poor, no study provided all the information this review sought.

Sample size

None of the reports contained a sample size calculation. In these studies the prevalence of *Plasmodium falciparum* malaria and antimalarial resistance markers varied between sample sets (63-100% and 0-100%, respectively), affecting the sample size required. As the prevalence of the condition of interest increases, the sample size required to accurately calculate sensitivity decreases, while the sample size required to accurately calculate specificity increases.

For a sample set containing only *Plasmodium falciparum* samples, assuming that index tests are 10% different in both sensitivity and specificity to the (theoretically) 100% accurate reference standard, with 80% power and 95% confidence intervals, sample size calculations (Figure 8) show that most studies were underpowered to determine sensitivity or specificity for at least one marker (Cruz et al., 2010, Moers et al., 2015, Pholwat et al., 2017, Alker et al., 2004, Andriantsoanirina et al., 2009, Chahar et al., 2017, Gan and Loh, 2010, Juliano et al., 2007, Juliano et al., 2009, Juliano et al., 2008, Kengne et al., 2003).

Durand et al. (2000), Durand et al. (2002), Farcas et al. (2006), Mens et al. (2008), Muhamad et al. (2011), Sharma et al. (2016), Wilson et al. (2005b) were appropriately powered for both sensitivity and specificity for all markers.

Figure 8: Formulae for sample size calculations

Sensitivity

$$\text{true positives + false negatives} = \frac{1.96^2 \times (\text{sensitivity} \times (1 - \text{sensitivity}))}{\text{accuracy}^2}$$

$$\text{sample size} = \frac{\text{true positives + false negatives}}{\text{prevalence}}$$

Specificity

$$\text{false positives + true negatives} = \frac{1.96^2 \times (\text{specificity} \times (1 - \text{specificity}))}{\text{accuracy}^2}$$

$$\text{sample size} = \frac{\text{false positives + true negatives}}{\text{prevalence}}$$

Indeterminate test results

All included studies had sufficient data available to create 2x2 tables of test accuracy. Some reports and authors provided enough information to allow the creation of 3x3 tables including data on unknown or indeterminate test results. These can be found in Table 67 to Table 84. Where data is available to create a 3x3 table, Table 26 displays the sensitivity and specificity calculated using both 2x2 and 3x3 tables of results where they differ.

For most studies data were available for a 3x3 table but did not alter accuracy estimates (Pholwat et al., 2017, Kengne et al., 2003, Durand et al., 2002, Durand et al., 2000, Sharma et al., 2016, Juliano et al., 2009, Alker et al., 2004, Muhamad et al., 2011, Gan and Loh, 2010, Chahar et al., 2017). For five studies data were not available for a 3x3 table (Juliano et al., 2008, Farcas et al., 2006, Andriantsoanirina et al., 2009, Juliano et al., 2007, Wilson et al., 2005b).

Table 26. Comparison of sensitivity and specificity calculated using 2x2 and 3x3 data

Study	Gene	Marker	Index Test vs Reference Standard	2x2	3x3
Mens et al. (2008)	*pfdhps*	540	RT-PCR-MCA/FRET vs PCR-RFLP	100% (90.3-100.0)	97.3% (85.8-99.9)
				100% (87.2-100.0)	87.1% (70.2-96.4)
Cruz et al. (2010)	*pfdhfr*	51	RT-PCR-MCA vs Sequencing	100% (88.1-100.0)	87.9% (71.8-96.6)
				90.9% (58.7-99.8)	90.1% (58.7-99.8)
		59		86.2% (68.3-96.1)	73.5% (55.6-87.1)
				77.8% (40.0-97.2)	70.0% (34.8-93.3)
		108		96.8% (83.3-99.9)	81.1% (64.8-92.0)
				83.3% (35.9-99.6)	71.4% (29.0-96.3)
		164		no change	-
				100% (90.5-100.0)	82.2% (67.9-92.0)
		51	RT-PCR vs Sequencing	100.0 (86.8-100.0)	78.8% (61.1-91.1)
				90.0% (55.5-99.7)	81.1% (48.2-97.7)
		59		100.0% (88.1-100.0)	85.3% (68.9-95.0)
				no change	100% (69.2-100.0)
		108		25.0% (0.6-80.6)	27.0% (0.0-14.2)
				-	0.0% (0.0-41.0)
		164		no change	-
				100.0% (81.5-100.0)	40% (25.7-55.7)
Moers et al. (2015)[5]	*pfcrt*	76	PCR-PEXT-NALFIA vs RT-PCR	90.0% (55.5-99.7)	81.8% (48.2-97.7)
				100.0% (47.8-100.0)	83.3% (35.9-99.6)
	pfmdr1	86	PCR-PEXT-NALFIA vs Sequencing	no change	100% (63.1-100.0)
				85.7% (42.1-99.6)	75% (34.9-96.8)
		184		100.0% (0.63.1-100.0)	88.9% (51.8-99.7)
				no change	100% (59.0-100.0)
Moers et al. (2015)[6]	*pfcrt*	76	PCR-PEXT-NALFIA vs RT-PCR	82.1% (66.5-92.5)	72.7% (57.2-85.0)
				100.0% (89.7-100.0)	85.0% (70.2-94.3)
	pfmdr1	86	PCR-PEXT-NALFIA vs Sequencing	92.9% (76.5-99.1)	74.3% (56.7-87.5)
				100.0% (95.7-100.0)	86.5% (78.0-92.6)
		184		86.6% (76.0-93.7)	67.4% (56.5-77.2)
				100.0% (91.6-100.0)	91.3% (79.2-97.6)

Ineligible Studies

The reason recorded for the ineligibility of a report for this review was the first reason identified while screening reports. The main reasons (in order of frequency) that reports were ineligible for inclusion in this review, including reports where only the title was screened, were that the studies:

- were not diagnostic accuracy studies
- did not examine *Plasmodium falciparum* malaria
- were published before 1983
- did not compare at least two molecular methods
- did not examine antimalarial resistance markers

A list of the 20 ineligible studies, which would have been eligible had sufficient results data been made available before 11th August 2017, can be found in Table 85. Other potentially eligible studies have already been published since 11th August (Imai et al., 2017).

No results could be derived from Nair et al. (2002), comparing PCR-PEXT to sequencing for *pfdhfr* and *pfdhps*.

It was not clear if Gil et al. (2003) used both PCR-RFLP and sequencing to detect *pfcytb* in clinical isolates.

Purfield et al. (2003) (conference abstract) investigated RT-PCR compared to sequencing for *pfmdr1* SNPs, and RT-PCR (reference standard unclear) for *pfmdr1* copy number; they found 100% concordance between methods.

Wilson et al. (2005b) was eligible when comparing RT-PCR to ASPCR for *pfcrt*, but results for sequencing were not available.

Mishra et al. (2006) compared Polymerase Chain Reaction Single Strand Conformational Polymorphism (PCR-SSCP) to PCR-RFLP for *pfcrt* and reported 100% efficiency.

Carnevale et al. (2007) used Multiplex Ligase Detection Reaction Fluorescent Microsphere Based Assay (LDR-FMA) compared to PCR for *pfcrt*, *pfdhfr* and *pfdhps*, they reported a concordance of 82.2% and an interassay concordance of 90.4-93.1%.

Crameri et al. (2007) reported an overall consistency of >90% for most SNPs in *pfcrt*, *pfdhfr*, *pfdhps*, *pfmdr1* and an agreement of 63.9-100%, when comparing a rapid PCR-PEXT-based microarray to PCR-RFLP.

Zhang et al. (2008) compared multiplex PCR microarray to sequencing for *pfcrt*, *pfdhfr*, *pfdhps* and *pfmdr1*; they reported that 1.3% of genotypes positions had inconsistent results between tests.

Khairnar and Pillai (2009) (conference abstract) examined *pfcrt*, *pfcytb, pfdhfr, pfdhps* and *pfmdr1* with pyrosequencing (reference standard unclear) and described pyrosequencing as "reliable".

Steenkeste et al. (2009) examined another microarray compared to sequencing for *pfcrt*, *pfdhfr*, and *pfmdr1*. They reported sensitivity of 95.88% and specificity of 97.68 %.

Matrevi et al. (2010) compared Sequence Specific Oligonucleotide Probe Enzyme Linked Immunosorbent Assay (SSOP-ELISA) to PCR-RFLP for *pfcrt* and *pfmdr1*; results were not described.

Mugasa and Ranford-Cartwright (2010) (conference abstract) used another PCR-based microarray (reference standard unclear) to detect *pfcrt*, *pfcytb*, *pfdhfr* and *pfdhps*; results were not described.

Akter (2012) compared a multiplex RT-PCR to PCR-RFLP for detection of *pfcrt* and *pfmdr1* and found the two methods to be "equally proficient" and the RT-PCR better at detecting mixed alleles.

Daniels et al. (2012) used RT-PCR-HRM compared to sequencing to detect *pfcrt*, *pfcytb*, *pfdhfr*, *pfdhps*, *pfmdr1* and found "100% correspondence"

Kamau et al. (2012) compared RT-PCR to sequencing to detect *pfdhfr*, *pfdhps* and *pfmdr1*; they reported 100% sensitivity and specificity.

LeClair et al. (2013) optimised a Multiplex Ligase Reaction-Fluorescent Microsphere (LDR-FM) assay for detection of *pfcrt*, *pfdhfr, pfdhps* and *pfmdr1* compared to PCR-RFLP. Concordance between the two methods was 78-100%, most discrepancies were due to mixed alleles.

Morris et al. (2013) used PCR-RFLP for SNP detection and RT-PCR for copy number quantification. Different DNA extraction methods were compared. Extraction from RDTs and filter paper were "generally equal", simple elution was unsuccessful.

Nankoberanyi et al. (2014) compared LDR-FM to PCR-RFLP for *pfcrt* and *pfmdr1*; agreement between the assays was between 79-97%.

Thompson et al. (2015) (conference abstract) used RT-PCR-HRM to detect *pfcrt*. The reference standard was not described, neither were results.

Ndiaye et al. (2017) compared RT-PCR-HRM to PCR–RFLP to detect *pfdhfr*, they reported that "only HRM detected a low level of mixed allelic infections".

SWARs

SWAR 6: Measuring the time taken for activities in systematic reviews

No data is available for SWAR 6.

SWAR: Finding eligible studies efficiently

Searching any two databases, other than MEDLINE and EMBASE would return all 18 eligible studies. Searching any one database would return at least 16 of the 18 eligible studies. Searching reference lists of eligible studies did not return any additional studies.

Table 27. Source of eligible studies

	Durand et al. (2000)	Durand et al. (2002)	Kengne et al. (2003)	Alker et al. (2004)	Wilson et al. (2005b)	Farcas et al. (2006)	Juliano et al. (2007)	Juliano et al. (2008)	Mens et al. (2008)	Andriantsoanirina et al. (2009)	Juliano et al. (2009)	Cruz et al. (2010)	Gan and Loh (2010)	Muhamad et al. (2011)	Moers et al. (2015)	Sharma et al. (2016)	Chahar et al. (2017)	Pholwat et al. (2017)	Number of eligible studies	Number of unique eligible studies	Number of search results
MEDLINE	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓	17	0	5054
EMBASE	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓		✓	16	0	7086
BIOSIS	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓	✓		✓	✓	✓	✓	✓	16	0	4122
Science Citation Index	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	17	0	6047
Reference Lists	✓	✓		✓	✓		✓	✓	✓	✓									8	0	494
Authors of eligible studies and experts																			0	0	0

Discussion

Methods

Only one reviewer screened search results, extracted data from eligible studies and assessed the risk of bias and applicability concerns. This may have affected the quality of the review by introducing inaccuracies, but the risk, direction and size of effect of any inaccuracies is unclear. This will be checked when the review is updated by two reviewers.

Ineligible studies

One study has now been confirmed to be eligible and data available for inclusion (Morris et al., 2013). Correspondence with authors of six of the currently ineligible studies leads to hope that data will be shared and studies included in the proposed update and expansion to this review (Kamau et al., 2012, Gil et al., 2003, Carnevale et al., 2007, Nair et al., 2002, LeClair et al., 2013, Nankoberanyi et al., 2014). Data for the other 13 studies is still being sought. The effect of being unable to include these studies in this review is unclear.

Unpublished data

Without including unpublished data, this review would not include any information on any *pfk13* markers, would be missing any data on four of the six *pfmdr1* markers, and would be missing any data on the 436-437 haplotype of *pfdhps*. Also, each of these studies investigated a unique index test: data on these three tests could not otherwise have been included in this review. As no statistical synthesis was carried out, any imprecision or bias in these studies could not affect any statistical summary or comparison of tests.

Search results

This search returned a huge number of results to be screened; the search was not specific. It will be possible to improve the specificity of future searches for expansions and updates of this review based on the results of this search. For example, the databases need only be searched from the year 2000 onwards, and the comprehensive list of reference tests identified here can be used as additional search terms. Searches additional to those of bibliographic databases did not yield any additional eligible studies which they were expected to do had the bibliographic search not been sensitive.

Molecular markers

Each of the genes in which there are markers of interest were studied in at least one of the eligible studies, indicating that including all of these genes was the correct decision. However, genes not covered by this review such as *pfatp6* were included in some of the already eligible studies; it may have been a mistake not to include them as a part of this review as minimal additional work would have been required to extract the additional useful data.

Of the markers of interest, only one was not included in any of the eligible studies, however, additional markers in the same genes were included in eligible studies but not in this review. An updated review might include some or all of these additional markers (Ecker, 2012).

Many methods were designed to detect a particular antimalarial resistance mutation (such as *pfcytb* 268C), and may miss different non-synonymous mutations present at the same marker (such as *pfcytb* 268S or 268N) (Pholwat et al., 2017).

Index and reference tests

Although a complete list of index tests could not have been anticipated, the three main reference standards, used in 16 of the 18 studies (but not for all comparisons within these studies) could have been, and used to refine the search. However, this would have reduced the comprehensiveness of the

review. There is little replication of testing of index tests in different studies; without replication, confidence in the results is lower.

Study characteristics

Sampling method

Only Farcas et al. (2006) used consecutive recruitment of patients and samples, which is unlikely to have introduced bias or reduced applicability of the results. Other studies may have used case-control designs. However, this was not stated and so had to be classified as an unclear risk of bias for all studies. Directly selecting already genotyped samples may remove from the selection samples for which it is more difficult to detect the genotype, due to contaminants, mixed infections, or a low concentration of DNA. In addition, samples may have been chosen due to other known characteristics such as a parasitaemia detectable by microscopy which will eliminate samples with a low parasitaemia (Joanny et al., 2014, Zurovac et al., 2006, Mukadi et al., 2011, Gupta et al., 2010, McKenzie et al., 2003). If case-control designs were used this could have introduced a large bias artificially inflating the assessments of test accuracy, and reduced the applicability of the patients and samples to the review question (Whiting et al., 2013).

Laboratory

No papers described the laboratories in which the tests were conducted. Diagnostic and parasitology labs may have the most expertise and be able to get the tests to perform to their full potential accuracy, but may not carry out the majority of surveillance work.

Dates

No study dates were described but the date of the publication of results can be used as a reasonable proxy to determine the approximate age of the samples. Only 10 studies described the dates of sample collection. The quality of DNA is negatively impacted by longer duration of storage, and this may

introduce a small bias in some studies, reducing estimates of test accuracy (Bauer et al., 2003). Surveillance programs will examine samples quickly to produce up-to-date data, in real-life scenarios they will not be stored for prolonged periods.

Country of infection and study, and speciation

The origin of the samples is mainly of interest to distinguish returning travellers to non-endemic countries from residents in endemic countries, and to consider the species of *Plasmodium* that may be present.

Parasitaemia may be generally lower in returning travellers who will have no immunity to *Plasmodium* (Cruz et al., 2010, Durand et al., 2000, Durand et al., 2002, Farcas et al., 2006, Moers et al., 2015), and in residents of endemic countries who are asymptomatic, protected by developed immunity (Alker et al., 2004, Wilson et al., 2005b, Juliano et al., 2007, Juliano et al., 2008, Juliano et al., 2009, Crompton et al., 2010, Crompton et al., 2014). Studies including these patients (or not) are not at an increased risk of bias, but may find a lower accuracy of tests; results are perfectly applicable to the populations of interest who include these groups.

This review considered the presence of non-*falciparum Plasmodium* infections. Initially, the plan had been to exclude studies using samples from areas where *Plasmodium falciparum* was responsible for <90% of infections, unless a PCR-based technique for speciation had been carried out. However, while carrying out the review a more pragmatic approach was developed. As the tests will be required to differentiate between these species in real-life surveillance scenarios, all studies, regardless of species prevalence and speciation were included.

Studies including returning travellers may have included non-*falciparum* infections where speciation was not carried out by a reliable method (Durand et al., 2000, Durand et al., 2002). This may also be true of studies of endemic cases in areas where multiple species circulate (Muhamad et al., 2011, Chahar et al., 2017, Pholwat et al., 2017). Only (Pholwat et al., 2017) explicitly included different species and reported the results considering this. The results of this study are highly applicable when

speciation has not yet been carried out for returning travellers. The other studies may or may not have carried out speciation; if not they may have introduced a small or large bias reducing the appearance of accuracy of the tests by testing non-*falciparum* infections for *falciparum* genes. Without more information on these studies, it is not possible to consider applicability.

The countries in which the work was carried out are mostly non-endemic countries. This highlights the continued need for capacity building in endemic countries, where the majority of surveillance work will need to be carried out.

Eligibility criteria

The only study providing detailed eligibility criteria, Farcas et al. (2006), shows that the patients and samples selected are highly likely to be representative of those found in real clinical scenarios in non-endemic countries.

Diagnosis of malaria

All methods for diagnosing *Plasmodium* infection were specific, but may have been lacking in sensitivity, if samples with lower density infections were excluded from studies the sensitivity and specificity of the tests may be exaggerated to a small degree by spectrum bias (Whiting et al., 2013).

Patient demographics

Characteristics such as sex, age, and concomitant medications are mostly of interest due to the effect they may have on parasitaemia. Children under 5, pregnant women, and patients with HIV are all likely to have a much lower immunity to *Plasmodium* infection, exhibit symptoms and be diagnosed with a lower parasitaemia (Juliano et al., 2009, Juliano et al., 2008, Wilson et al., 2005b, Alker et al., 2004, Juliano et al., 2007, Chiu et al., 2015, Osterbauer et al., 2012, Pava et al., 2016, Ogbu et al., 2015, Fana et al., 2015). Samples taken at diagnosis will have a lower concentration of parasite DNA and this may reduce the accuracy of the test for these populations. These results are highly applicable to some of the patients of interest in this review question.

Sample source

All studies that reported the source of blood used peripheral, finger prick, or venous blood, which is unlikely to have any effect on estimates of test accuracy and is highly applicable to the review question.

Parasitaemia

Only Farcas et al. (2006) designed their study in a way that ensured that parasitaemias of samples in the study were representative of parasitaemias of samples relevant to their clinical question. Cruz et al. (2010) provided unpublished data including individual parasitaemias, both Wilson et al. (2005b) and Juliano et al. (2007) provided averages and ranges in their published reports; these were all wide in range and representative of the parasitaemias one would expect to encounter in real clinical scenarios. Other studies may not have included representative samples, introducing a bias, likely in favour of overestimating accuracy.

Multiplicity of infection

Multiplicity was of interest as infections with higher multiplicity may be more likely to have mixed populations of both sensitive and resistant alleles (Kyabayinze et al., 2008, Chang et al., 2016, Nag et al., 2017). No information on multiplicity was provided.

Sample storage

Storage conditions can have a large effect on the quality of DNA whether it has been extracted from whole blood or not (Schwartz et al., 2015, Hsiang et al., 2010). DNA quality will be higher in samples collected more recently, those stored at lower temperatures in sealed containers, and those stored with desiccant. Test accuracy may be higher for samples that have higher quality DNA. Resources available vary hugely, and tests will need to be accurate in all settings. Kengne et al. (2003) is the only report that makes clear that the storage conditions were sufficient to protect the DNA. DNA in other

studies may have had more chance to degenerate, reducing the appearance of accuracy of the test, probably by a small amount.

DNA extraction

Less efficient methods of DNA extraction (ethanol precipitation, chelex and phenol-chloroform in particular) will lead to samples with lower concentrations of DNA, this may have a small or large effect, making tests appear less sensitive (Zainabadi et al., 2017, Morris et al., 2013, Psifidi et al., 2015).

Number of samples and participants

The number of participants was not clearly reported for most papers; the number of samples was reported. The inclusion of multiple samples from the same participants is unlikely to have introduced any bias as these samples were taken before and after treatment and chosen due to treatment failure, rather than because of the genotype that had been detected. The possible use of the same samples in more than one study (most likely in Juliano et al. (2008), Juliano et al. (2007) and Juliano et al. (2009) may have introduced a bias if samples were chosen due to their having been successfully genotyped previously. Exclusion of samples for which it has been more difficult to establish genotype may have introduced a spectrum bias causing a small inflation in estimates of test accuracy,

Index test

13 different index tests were studied, where the same tests were studied there were differences in conduct. The variety of promising index tests is positive, but the lack of replication in studying these tests is a drawback. Few tests were suited to conduct in low resource environments and tests PCR-PEXT-NALFIA and TAC required equipment that was not commercially available.

Reference standard

There is no established gold-standard for detection of antimalarial drug resistance markers, few studies included in this review compare the reference standards to each other, and do not for the same molecular markers. Shortcomings in sensitivity and specificity are more likely to be

acknowledged of older techniques as newer technologies appear to be more sensitive and specific. However, there is no way to confidently determine the accuracy of any of the reference tests. An increasing understanding of the complexity of *Plasmodium* infections (although detection of different populations is also imperfect) suggests that current antimalarial resistance marker detection methods may still be missing minority resistance alleles (Anderson et al., 2000, Tanabe et al., 2015). This has been demonstrated by comparisons of parasite populations *in vivo* before and after treatment, and *in vitro* before and after adaptation to culture, which show that different, previously undetected, populations are revealed as selection pressures alter (White et al., 2016b, Sisya et al., 2015, van Schalkwyk et al., 2013).

Improving sensitivity is likely to result in reduced specificity. In addition, the paradox that more sensitive and specific tests will appear less accurate when compared to less sensitive and specific reference tests means that establishing the most accurate test is difficult (Wang et al., 2017). From the results of this review, it may be that PCR-RFLP and ASPCR are likely to be more specific and less sensitive, and RT-PCR and sequencing are likely to be more sensitive and less specific. PCR-MSS-HTA has been much less studied, in this review comparisons suggest that it may be more sensitive than ASPCR and approximately equal in sensitivity and specificity to RT-PCR (Juliano et al., 2008, Juliano et al., 2007).

Test interpretation

The conduct and interpretation of both index and reference tests were incompletely described by all studies. Little enough information was provided that the risk of bias and applicability was judged to be unclear for most studies (Whiting et al., 2013).

Funding

Funding from industry has been shown to be correlated with a bias in favour of index test accuracy, this may affect the results of Durand et al. (2000), Farcas et al. (2006), Juliano et al. (2008) and Juliano et al. (2009) which received industry funding and/or assistance (Canestaro et al., 2017).

Risk of bias and applicability concerns

QUADAS-2 was used to assess the methodological quality of eligible studies. The vast majority of studies were at an unclear risk of bias, and unclear applicability, for domains patient selection, index test, and reference standard. Only flow and timing was considered to be at low risk of bias for most studies.

Patient Selection

All studies other than Farcas et al. (2006) are at an unclear risk of bias for patient selection. This risk of bias could be low or high, small or large, and bias the results in either direction, increasing or reducing estimates of test accuracy. The applicability of the studies (other than Farcas et al. (2006)) could be low or high. Farcas et al. (2006) is very unlikely to have introduced any bias through patient selection, and results are highly applicable to the review question.

Index Test

All studies other than Durand et al. (2000) and Durand et al. (2002) are at an unclear risk of bias which could be low or high, small or large, and bias the results in either direction, increasing or reducing estimates of test accuracy. The applicability could be low or high. Durand et al. (2000) and Durand et al. (2002) are both at a low risk of introducing of any bias. The applicability of the index test conduct and interpretation to this review question is high.

Reference test

Six studies were at a low risk of bias. The risk of bias in Durand et al. (2000) is particularly low. The other five studies have a low risk of bias, but it was unclear whether the thresholds used were pre-specified – there is a low risk that a bias (which could be small or large) artificially inflated estimates of test accuracy. These six studies were also at a low level of concern regarding applicability. The risk of bias in the other 12 studies could be low or high, small or large, and bias the results in either direction, increasing or reducing estimates of test accuracy. The applicability could be low or high.

Flow and timing

More information was available to answer signalling questions for this domain. For all studies it is likely (but not explicitly stated) that one sample taken underwent processing such as DNA extraction once and then was used for both tests without a large delay in between. 13 studies were at a low risk of bias, any bias introduced is likely to be small but the direction is unclear. If there was a delay between samples being tested, this would likely favour the first test where the DNA would be more stable before a lengthy storage. However, as the information on interval between tests was not described, we do not know which test was carried out first.

Three studies were at a high risk of bias as not all samples were included in the analysis (Wilson et al., 2005b, Juliano et al., 2008, Andriantsoanirina et al., 2009). The size of the bias this is likely to create is large, and artificially inflating the estimates of test accuracy. Similarly, two studies were at a high risk of bias as not all samples received the reference test, and not all samples were included in the analysis (Alker et al., 2004, Juliano et al., 2007). The size of the bias this is likely to create is also large, inflating the estimates of test accuracy.

Summary

Overall Durand et al. (2000) and Durand et al. (2002) are at a low risk of bias, if there is a bias it is likely small, and inflating estimates of test accuracy. Farcas et al. (2006) is classified similarly as having a low risk of bias, the size of any bias would be small, and favour a higher test accuracy. For the other 15 studies there is too little information available to state the overall risk, size and direction of biases. Potentially the risk of bias in all of these is high, large in size, and more likely to favour inflated estimates of test accuracy. Results of these studies will be considered with some uncertainty as to their reliability, however poor reporting does not necessarily correlate with poor practice (Soares et al., 2004).

Sensitivity and specificity

***pfcrt* 76**

This review has the most confidence in the accuracy results of Farcas et al. (2006) (RT-PCR) and Durand et al. (2000) (PCR with molecular beacons), both of which have high sensitivity and specificity estimates with relatively small 95% CI. Other tests appear to be similarly accurate (RT-PCR-HRM and LAMP), although with unclear bias and applicability, the results of these studies may be less reliable. Interestingly, with two different sample sets, and also with a different reference test, the estimates of sensitivity of PCR-DIG-HTA by Juliano et al. (2009) vary; the accuracy of this index test may be particularly dependent on some characteristic that differs between the samples from Malawi and those from Madagascar. Estimates of accuracy are lower for PCR-PEXT-NALFIA.

If a RT-PCR machine is available, this may be the quickest, most high-throughput and accurate method. Otherwise, compared to other methods, LAMP requires little time and no specialist equipment, and has already been successfully developed for diagnosis of malaria in the field, researchers in low resource setting may wish to use LAMP (Aydin-Schmidt et al., 2014, Hopkins et al., 2013).

***pfcrt* 72-76**

The risk of bias and applicability concerns are unclear for all studies investigating this haplotype. Either RT-PCR-HRM or TAC may be the most accurate method, both require specialist equipment and are not suitable for low resource settings. However, as *pfcrt* 72 is the marker of most interest, researchers for whom RT-PCR and TAC are not viable options may wish to genotype only this marker using PCR with molecular beacons or LAMP as described above.

***pfcytb* 268**

The risk of bias and applicability concerns are unclear for all studies investigating this genotype. Either RT-PCR-HRM or TAC may be the most accurate method, both require specialist equipment and are not suitable for low resource settings.

***pfdhfr* 51**

The risk of bias and applicability concerns are unclear for all studies investigating this genotype. Either RT-PCR-HRM or TAC may be the most accurate method with the highest and most precise estimates of sensitivity and specificity. However, both require specialist equipment and are not suitable for low resource settings. If a PCR machine is available, ASPCR could be expected to be sensitive, but not specific.

***pfdhfr* 59**

The risk of bias and applicability concerns are unclear for all studies investigating this genotype. Either RT-PCR-HRM or TAC may be the most sensitive methods, however, specificity is the highest with RT-PCR-HRM, followed by RT-PCR. If a RT-PCR machine is available, RT-PCR-HRM may be the quickest, most high-throughput and accurate method. Otherwise, if equipment for TAC is available, this may be the next best option. If a PCR machine is available, ASPCR is also an option but may be much less sensitive and specific.

***pfdhfr* 108**

This review has the most confidence in the accuracy results of Durand et al. (2000) (PCR with molecular beacons), which has high sensitivity and specificity estimates with relatively small 95% CI. Other tests appear to be similarly sensitive (ASPCR, RT-PCR-HRM and TAC). If a PCR machine is available, PCR with molecular beacons is the most sensitive method to choose. If a RT-PCR machine or reagents for TAC are available, these methods may be similarly sensitive with a higher throughput. RT-PCR-HRM has a similar specificity to PCR with molecular beacons, if a RT-PCR machine is available, this higher throughput option may be preferred.

***pfdhfr* 164**

The risk of bias and applicability concerns are unclear for all studies investigating this genotype. TAC may be the most sensitive method, however, specificity is the highest with TAC, PCR-MSS-HTA or RT-PCR-HRM. If the equipment for TAC is available, this may be the most accurate method to use. If an RT-PCR machine is available, this would be the next most specific method, although perhaps lacking in specificity. If a PCR machine is available, PCR-MSS-HTA may be preferred, however, this method also requires the resources to handle radiolabelled probes.

***pfdhps* 436**

The risk of bias and applicability concerns are unclear for all studies investigating this genotype. RT-PCR-HRM is the only method with an estimate of sensitivity. Although the point estimate for specificity is higher with RT-PCR, the CI show that RT-PCR-HRM may be more specific. Overall, RT-PCR-HRM is likely to be the most accurate method, but requires the use of a RT-PCR machine.

***pfdhps* 437**

The same two studies investigated *pfdhps* 437; the risk of bias and applicability concerns are unclear for both studies. RT-PCR-HRM has the lowest point estimates, but the smallest CI, RT-PCR could be

the most accurate, but also could be the least accurate. RT-PCR-HRM may be the safest method (in terms of accuracy) to choose, but requires the use of a RT-PCR machine.

***pfdhps* 436-437**

The risk of bias and applicability concerns are unclear for Pholwat et al. (2017) haplotyping 436-437. Only an estimate of sensitivity could be calculated for TAC, this was a higher sensitivity than RT-PCR or RT-PCR-HRM for either 436 or 437 individually. However, specificity could not be calculated for TAC and could be for methods genotyping 436 or 437 individually. Depending on the resources available, either TAC or RT-PCR-HRM may be preferred, but both may be difficult to use in low resource settings.

***pfdhps* 540**

The risk of bias and applicability concerns are unclear for all the studies examining *pfdhps* 540. Sensitivity may be highest for TAC, but specificity could not be calculated. Point estimates of sensitivity and specificity are highest for RT-PCR and RT-PCR-HRM, but CI are wide; these methods could be the most or the least sensitive. CI are narrower for RT-PCR-MCA/FRET. If sensitivity is critical, and specificity less so, TAC may be the best method to choose. If a RT-PCR machine is available, RT-PCR-MCA/FRET may be the most sensitive method to choose, and RT-PCR-HRM the most specific method. All methods require specialist equipment.

***pfdhps* 581**

The risk of bias and applicability concerns are unclear for both the studies examining *pfdhps* 581. Sensitivity and specificity may be similar for RT-PCR and TAC; TAC has narrower CI and is a safer choice. However, if equipment for TAC is not available, and an RT-PCR machine is, RT-PCR may provide accurate results.

***pfdhps* 613**

The risk of bias and applicability concerns are unclear for both the studies examining *pfdhps* 613. Only specificity could be calculated, it may be similar for RT-PCR and TAC; TAC has narrower CI and is a safer

choice. However, if equipment for TAC is not available, and an RT-PCR machine is, RT-PCR may provide accurate results.

***pfk13* 458, 493, 539, 543, 561, 580**

The risk of bias and applicability concerns are unclear for Pholwat et al. (2017), the only study examining these six markers in *pfk13*. Specificity was calculated for TAC for all six markers, TAC shows a high specificity for all. Sensitivity was calculated for *pfk13* 561, the point estimate was high, but CI are very wide. Resource limitations may not permit the use of TAC in many settings. However, in the absence of other studies testing other molecular methods, TAC is the only method included in this review for these markers.

***pfmdr1* 86**

The risk of bias and applicability concerns are unclear for the three studies examining molecular methods for detecting this marker. If equipment is available to use TAC, this may be the most accurate method to choose. If not, either RT-PCR-HRM or PCR-PEXT-NALFIA may be the most accurate, but both also require specialist equipment. PCR-PEXT-NALFIA may be slightly less accurate than RT-PCR-HRM, but does not require a RT-PCR machine.

***pfmdr1* 184**

The risk of bias and applicability concerns are unclear for the two studies examining molecular methods for detecting this marker. If equipment is available to use TAC, this may be the most accurate method to choose. If not, PCR-PEXT-NALFIA also shows high sensitivity and specificity, but with larger CI. Both require specialist equipment that may not be available in a low-resource setting.

***pfmdr1* 1034, 1042**

The risk of bias and applicability concerns are unclear for Pholwat et al. (2017), the only study examining molecular methods for detecting either of these two markers. If equipment is available to

use TAC, this may be a highly specific method to use for both markers. Sensitivity could not be calculated for *pfmdr1* 1034, for *pfmdr1* 1042; this method may have low or high sensitivity.

***pfmdr1* 1246**

The risk of bias and applicability concerns are unclear for the two studies examining molecular methods for detecting *pfmdr1* 1246. If equipment is available to use TAC, this may be a highly sensitive and specific method to use. Sensitivity may be slightly higher using PCR-PEXT-NALFIA; specificity may be lower. However, both require specialist equipment and it may not be possible to conduct them in low resource settings.

***pfmdr1* copy number**

The risk of bias and applicability concerns are unclear for Muhamad et al. (2011), the only study examining a molecular method for detecting this marker. If equipment is available to use RT-PCR, this may be a moderately to highly sensitive and specific method to choose. However, an RT-PCR machine is required.

Registration of studies

Prospective registration allows wider scrutiny of trial protocols, which may lead to improvements in study designs. It prevents unnecessary or unintentional duplication, and enables planned replication. It improves the findability of studies, the compatibility of data from different studies, and the information available for thorough critical appraisals. Along with the publication of protocols it assists the identification of biases such as publication bias and outcome switching (Silagy et al., 2002, Page et al., 2014). None of the studies eligible for this review, or studies eligible subject to supply of additional results are registered; registration of Moers et al. (2015) will be carried out retrospectively.

This review was hindered by a search strategy lacking in specificity, a dearth of basic information about study design and conduct, sub-optimal study design, variations in conduct preventing statistical

synthesis, and an absence of detailed study results. All of these problems could be reduced or eliminated by the use of prospective study registration; a STARD initiative is now underway to improve registration of diagnostic accuracy studies (Korevaar et al., 2017).

Reporting of studies

None of the studies eligible for this review, or studies eligible subject to supply of additional results were reported following the current gold-standard for reporting of diagnostic test accuracy studies; STARD 2015 (Bossuyt et al., 2015). There are focused guidelines for some specialities and other guidelines such as SPIRIT 2013 (writing protocols) and TIDieR (describing interventions) which may aid precise and complete reporting of diagnostic accuracy studies (Equator Network, 2017, Chan et al., 2013, Hoffmann et al., 2014, Hoffmann et al., 2017).

If all 38 studies had been reported according to STARD 2015 guidelines this review could have more quickly screened reports and extracted data from them. This review would also have been able to assess the risk of bias and applicability concerns of studies more usefully as “low” or “high” and take this into account when considering results, rather than a majority of domain assessments being “unclear”. This review could also have included up to 38 studies, rather than 18 (15 without seeking unpublished data), as STARD 2015 requires “Cross tabulation of the index test results (or their distribution) by the results of the reference standard”. The level of detail provided by most reports is insufficient to enable other researchers to replicate the molecular methods used, or corroborate the results determined, or to use these methods in a surveillance program.

These problems may be in part due to the fact that diagnostic accuracy studies have traditionally not been accorded the same importance as treatment intervention trials, and are often tacked on to larger studies as an afterthought.

The positive response of authors to requests for unpublished results may indicate that efforts to obtain other unpublished details may be successful.

Sample sizes

The lack of reported sample size calculations does not necessarily indicate that these were not made by researchers. Studies may have been underpowered due to different test sensitivities or different resistance allele prevalence than expected, under powering of diagnostic studies is common (Bachmann et al., 2006). However, the effect of underpowered studies is the same; very wide CI and a lack of precision in the estimates of test sensitivity and/or specificity. Most of the studies were underpowered for a least one of the markers they were investigating and this has negatively impacted the ability of this review to precisely estimate test accuracy and identify the most accurate methods.

Indeterminate results

Indeterminate reference test results could not be taken into account in the estimates of sensitivity and specificity, however, indeterminate index results can be included in the denominator when calculating both sensitivity and specificity. 13 studies included sufficient data to compare 2x2 tables of test accuracy with 3x3 tables of test accuracy, for three of these studies the presence of indeterminate index test results altered the estimates of sensitivity or specificity. The differences in point estimates varied from 0.1% to 60%. The impact of indeterminate results on estimates of accuracy, for studies where indeterminate results are not available, is unclear, and may be large. Considering this, the update to this review may consider revising their eligibility criteria to require the results to construct a 2x3 table. Perhaps the next update to the STARD guidelines could require the inclusion of indeterminate results in the cross-tabulation of results.

Ineligible studies

Without carrying out data extraction for each of the ineligible studies, which is beyond the resources of this review, it is not possible to thoroughly summarise the ineligible studies and consider the impact their inclusion may have had on the results of this review. A brief investigation of each of these study reports shows that the studies were, in general, so vaguely reported that it is not useful (or sometimes possible) to extract just test methods and molecular markers and consider these in relation to the results. The majority of markers considered were already included in this review with data from other studies. Nair et al. (2002) and Crameri et al. (2007) both investigated *pfdhfr* A16V, an important marker of resistance to cycloguanil and pyrimethamine, which was not covered by any of the studies eligible for this review, inclusion of this data in particular would be useful. Most of the index and reference tests used in eligible studies were the same as those included in this review. Some studies considered other tests not covered by this review and inclusion of these would improve the comprehensiveness of this review:

- PCR-SSCP (Mishra et al., 2006)
- LDR-FMA (Carnevale et al., 2007, LeClair et al., 2013, Nankoberanyi et al., 2014)
- Microarrays (Crameri et al., 2007, Zhang et al., 2008, Steenkeste et al., 2009, Mugasa and Ranford-Cartwright, 2010)
- Pyrosequencing (Khairnar and Pillai, 2009)
- SSOP-ELISA (Matrevi et al., 2010)
- Multiplex RT-PCR (Akter, 2012)

Responsiveness of researchers to email requesting additional information have been positive, and it is hoped that in the updated and expanded review that researchers will provide sufficient information for these studies to be included.

SWARs

SWAR 06: Measuring the time taken for activities in systematic reviews

SWAR 6: Measuring the time taken for activities in systematic reviews, was started as a part of this review, to contribute to a database about the time taken to conduct different parts of systematic reviews (Burrow, 2016). However, it wasn't possible to maintain the frequency and accuracy of reporting that will provide useful data, this SWAR needs to be modified to improve usability.

SWAR: Finding eligible studies efficiently

This review could have been carried out much more efficiently. By searching just two databases the number of studies screened to find all eligible studies could have been reduced from 22,803 to 9,176. 17 of the 18 eligible studies could have been found by searching just one database returning 5,054 results. This proposed SWAR will be submitted to the Northern Ireland Network for Trials Methodology Research SWAR Repository (Northern Ireland Network for Trials Methodology Research, 2017).

Limitations

This review is limited by the use of only one reviewer which may have impacted accuracy. It includes tests for only some molecular markers, others are of interest in surveillance programs. Potentially eligible studies were excluded due to missing results data, inclusion of these studies may contribute valuable data, but including these studies without this data could lead to bias in estimates of accuracy (Shinkins et al., 2013). Only methods with some published reported of diagnostic accuracy were included, accurate methods may have been tested but the results not published, or not tested formally in diagnostic accuracy studies. Gaps in reporting of studies make interpretation of study results unclear. Known deficiencies in study designs increase the risk of bias in included studies. No studies examining economic considerations, adverse effects or patient important outcomes were included.

Recommendations

The inclusion of all potentially eligible studies in the update to this review will add useful data, but gaps in research remain. High quality, well reported diagnostic studies are required. In particular, markers for the most clinically relevant markers currently (*pfcrt* 76, *pfmdr1* 86, 184, *pfhpfr* 51, 59, 108, 164 and *pfdhps* 436, 437, 540, 613) and those likely to be useful in the future (*pfk13* 580) should be examined. The focus should be on accurate molecular methods suitable for high throughput in high resource settings, and fast simple processing in low resource settings, although other characteristics and impacts of tests should be considered; studies included in this review did not consider economic impacts, potential harms of misdiagnosis or patient relevant outcomes (Mustafa et al., 2017, ACTwatch Group et al., 2017a, Roberts, 2017, Lord et al., 2006). An example of a high quality study, unlikely to introduce bias, highly applicable to some diagnostic and surveillance scenarios, itself providing useful surveillance data, powered sufficiently to produce a reasonably conclusive results has been adapted from an assignment and is provided in the appendices. In the future, when these tests are considered as part of the diagnostic pathway, rather than only surveillance, their position(s) must be established.

Dissemination

Interim results have already been presented at the University of Oxford Tropical Medicine Seminar and at a Kellogg College Colloquium. This manuscript of this review will be circulated via relevant parasitology and malariology list-servs and by email to colleagues carrying out surveillance of molecular markers of antimalarial resistance. The update to this review is already underway. In addition to the above, the protocol and full results of the review will be published in a scientific journal and circulated among researchers working in surveillance. Results will be presented at relevant scientific conferences. Social media will be used to communicate scientific and lay summaries.

Conclusions

This is the first systematic review of this subject: a systematic review of diagnostic test accuracy of all molecular methods for detecting antimalarial resistance genotypic markers.

This review found that the most accurate test for most molecular markers is unclear. Few tests are suitable for conduct in low resource environments where the majority of surveillance may be required. Hopefully researchers will use these findings to discard any clearly suboptimal methods and adopt more accurate methods; reducing research waste, improving the coverage and accuracy of antimalarial resistance surveillance, and translating into a reduced number of cases of malaria, and fewer deaths from malaria.

An update to this review is already under way, including more studies with currently unpublished data, more molecular markers of antimalarial resistance and more molecular methods for detection. In addition, two reviewers are undertaking this update in order to ensure that the review is carried out to a high standard, and suitable for publication in a scientific journal.

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Appendices

PRISMA checklist

Table 28. PRISMA Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	11-16
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	17
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	18
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	18-20
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	21
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	21-22
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	23-24
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	25-27

Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	26-27
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	27-28
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	28
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	28-29
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	na
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	na
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	31-32
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	33-44
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	45-48
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	49-69
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	na
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	45-48
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	na
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	74-93
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	93
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	94-95
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	10

Systematic Review Protocol

Sensitivity and specificity of molecular methods for detecting markers of antimalarial drug resistance in clinical samples of *Plasmodium falciparum*: protocol for a systematic review

Registration

In accordance with PRISMA-P guidelines, this systematic review protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO) on 30th January 2017 and was last updated on 28th July 2017 (registration number CRD42017056249) (CRD, 2016, Shamseer et al., 2015).

Amendments

All protocol amendments will be listed with the date, a description of the change, and the rationale in the published systematic review and in the registration in PROSPERO.

Support - Sources, Sponsor, Role of sponsor or funder

No funding or sponsorship has been received for this systematic review.

Competing interests

Rebekah Burrow is an author of a report of a potentially eligible study.

Availability of data and materials

The datasets supporting the conclusions of the systematic review article will be included within the article and in the additional files.

Consent for publication

Consent for publication is not applicable as no individual person's data is contained in the paper.

Abstract

Background

Each year infection with *Plasmodium* causes millions of clinical cases of malaria, and hundreds of thousands of deaths. Resistance to different antimalarial medications continues to develop and spread, threatening effective prophylaxis and treatment. Monitoring of resistance is required to inform health policy and preserve effective antimalarial drugs; molecular methods can be used to determine likely parasite susceptibility. However, there is no consensus on the most accurate methodologies, and large variation exists in practice. The goal of this systematic review is to identify the sensitivity and specificity of each molecular method for detecting any antimalarial drug resistance marker.

Methods

We will include diagnostic accuracy studies published between 1983 and 2017 that examine blood samples from patients diagnosed with, or suspected of having *falciparum* malaria, for at least one established marker of antimalarial drug resistance. All studies comparing at least two molecular methods will be included. We will search MEDLINE, EMBASE, BIOSIS, and Science Citation Index. Independently, one reviewer will screen all results for inclusion; a second reviewer will screen 10% of all results and 100% of results judged to be eligible by the first reviewer. Two independent reviewers will extract data and evaluate the methodological quality of included studies. We will carry out a meta-analysis and use statistical methods to compare results from homogenous studies. We will use narrative to synthesise and compare results of heterogeneous studies.

Discussion

This review will help to identify sub-optimal molecular methods for antimalarial marker detection which can be discontinued, and identify more sensitive and specific methods which can be adopted. More sensitive and specific detection of drug resistance can be used to improve the breadth and accuracy of surveillance; identify previously undiscovered areas of antimalarial resistance and

sensitivity, improve the precision of estimates of the prevalence of resistances, and improve our ability to detect smaller changes in these patterns. Higher quality evidence generated by more accurate and detailed surveillance could be used to inform guidelines on the use of antimalarial drugs, leading to better outcomes for more patients.

Systematic Review Registration

PROSPERO CRD42017056249

Keywords

Malaria – Antimalarials – Resistance – Diagnosis – Molecular – Systematic Review – Protocol

Introduction

Infection with *Plasmodium* caused 148-304 million clinical cases of malaria, and 235,000-639,000 deaths in 2015 (WHO, 2016c). Malaria is often treated effectively with an artemisinin-based combination therapy (ACT) (Sow et al., 2016, Toure et al., 2016, Pregact Study Group et al., 2016). However, some *Plasmodium* parasites are resistant to other antimalarial chemotherapies and responsible for increased mortality (Trape et al., 1998, Korsinczky et al., 2000, Nosten et al., 2000, Bousema et al., 2003, Mullick et al., 2011).

ACTs and artemisinins are the World Health Organization (WHO) recommended first-line treatments for most patients with uncomplicated *falciparum* malaria, or with severe malaria (WHO, 2015b). However, resistance to artemisinins has recently emerged in the Greater Mekong sub-region, linked to mutations in the *pfkelch13* gene (*pfk13)*, with a phenotype of delayed parasite clearance which may lead to treatment failure (Ariey et al., 2014, Ashley et al., 2014, Tun et al., 2015, Amaratunga et al., 2016, Thanh et al., 2017). Failure of ACTs may be due to other factors, such as resistance to partner drugs, and resistance to ACTs may be evolving in other regions (Venkatesan et al., 2014, Lu et al., 2017, Mukherjee et al., 2017). Efforts to eliminate malaria have been intensified in the Greater Mekong sub-region (WHO, 2016b) and world-wide surveillance of artemisinin resistance has been prioritised (World Health Organisation, 2016).

However, a minority of patients with malaria receive WHO recommended ACTs; most patients receive either no antimalarial drug, or a different antimalarial, to which resistance (with a phenotype of treatment failure) may already be widespread (Littrell et al., 2014, WHO, 2016c, WWARN, 2016, ACTwatch Group et al., 2017b).

Chemoprevention for high-risk groups in endemic countries often uses a combination of non-artemisinins; increasing resistance threatens efficacy (Meremikwu et al., 2012, Geiger et al., 2014, Muanda et al., 2015, Cisse et al., 2016).

For travellers to endemic countries, chemoprevention is recommended. Resistance to mefloquine has already developed (Price et al., 2004). Although some cases of resistance to atovaquone have been reported, resistance has not spread (Jacquerioz, 2015, Goodman et al., 2016).

Mass drug administration (MDA) programs have been used with mixed success in the past, and are currently being trialled. The WHO recommendations are to use a drug combination for MDA different to that for first-line treatment (Poirot et al., 2013, University of Oxford, 2013, WHO, 2015c).

Effective prophylaxis and treatment are essential for control of malaria and eventual elimination, but the emergence and spread of drug resistant parasites is a great impediment to achieving these goals. In the future, diagnosis and treatment will be supplemented by add-on point-of-care tests for detection of antimalarial resistances, as it is for other infectious diseases (Steingart et al., 2014). Until these diagnostics are developed and implemented widely to allow individualised treatment, surveillance will remain key to determining the chemoprevention and treatments most likely to be effective. Continuous global monitoring of therapeutic efficacy and drug resistance is required to detect epidemiological patterns of parasite susceptibility to all antimalarial drugs in order to inform malaria prevention and treatment policies (Plowe, 2003, Sibley et al., 2008, WHO, 2010).

In addition to identifying emerging resistance, and altering policy to ensure that effective antimalarials are recommended, reductions in resistance can also be detected. Fitness costs incurred by parasites with mutations conferring resistance, and antimalarial drugs exerting opposite selective effects, will contribute to the re-emergence of parasites sensitive to previously discarded antimalarials, which can then be redeployed (Ord et al., 2007, Humphreys et al., 2007, Sisowath et al., 2009, Kublin et al., 2003, Laufer et al., 2006, Lucchi et al., 2015). Reducing the use of ACTs by utilising other effective antimalarials, should slow the emergence and spread of resistance to ACTs and preserve them as effective antimalarials (Dondorp et al., 2010, Maude et al., 2010, Klein, 2013).

Historically, parasite susceptibility was determined using clinical trials or observational studies. In addition, for a limited number of clinical samples, therapeutic efficacy could be tested through *ex vivo*

and *in vitro* culture methods. More recently developed molecular methods offer many advantages over both of these; reduced patient burden, reduced requirement for staff with clinical and trial expertise, reduced delay to results, and increased throughput (Scientific Group on Resistance of Malaria Parasites to Drugs, 1965, WHO, 1973, Kublin et al., 1998, Plowe, 2003, Abdul-Ghani et al., 2014).

Molecular methods are able to detect single nucleotide polymorphisms (SNPs) and copy number variation associated with resistance of *P. falciparum* to many antimalarial drugs. The correlation between genotype and phenotype is mixed in strength for different molecular markers, so the clinical relevance of these molecular markers varies.

For example, when the T76K mutation is present in the *Plasmodium falciparum chloroquine resistance transporter* (*pfcrt*) gene, almost all patients fail to respond adequately to chloroquine treatment (Wellems and Plowe, 2001, Ecker et al., 2012). However, combinations of mutations in the *dihydropteroate synthase* (*pfdhps*) and *dihydrofolate reductase* (*pfdhfr*) genes are sometimes highly predictive of sulphadoxine-pyrimethamine (SP) treatment failure (East Africa) and sometimes not (West Africa) (Mockenhaupt et al., 2005, Alker et al., 2008, Mbacham et al., 2010, Warsame et al., 2017).

Factors other than one mutation may affect the presence and level of resistance, including other known mutations (such as *pfcrt* C72S) or as yet unknown genetic mutations conferring some degree of resistance directly, other parasite characteristics (including parasitaemia and virulence), host immunity, and interactions between parasite and host (Sherman, 1998, Grech et al., 2006, Schneider et al., 2012, Klein, 2013, Mantel et al., 2013). In spite of these issues, genotyping of antimalarial resistance markers is the surveillance method used most often and by most research groups (WWARN, 2017).

There is a lack of a standardised protocol for detecting resistance via molecular markers, with variation in sample source, collection and storage, DNA extraction, methods for detection, and analysis of

results of mutations associated with resistance (WHO, 2010). Some attempts have been made to standardise some methods for some markers, but without ascertaining the optimum method through a review of the available diagnostic accuracy studies (WWARN, 2014).

It seems likely that there is variation in the accuracy of different molecular methods for detecting antimalarial drug resistance. For example, Ndiaye et al. 2013 (Ndiaye et al., 2013) reported determining *pfdhfr* N51I, C59R and S108N for 416/416 (100%) of samples, while Ojurongbe et al. 2011 (Ojurongbe et al., 2011) determined a genotype for 39/79 (49%) of samples. Some differences may be due to patient and sample characteristics, such as parasitaemia, but it seems likely that test performance also has a role.

A systematic review comparing existing methods for genotyping molecular markers should identify many of the methods in use, and the sensitivity and specificity of each method for detecting each drug resistance marker (Leeflang, 2014). Any methods found to be sub-optimal in a particular setting could be discontinued from use, and replaced by the more sensitive and specific methods. Use of more sensitive and specific methods would be more efficient, using fewer resources to identify markers in the same number of samples. It would also result in fewer gaps in surveillance, and improved accuracy of surveillance. This will identify previously undiscovered pockets of antimalarial resistance, improve the accuracy and precision of estimates of known resistance, and improve our ability to detect smaller changes in these patterns.

It seems likely that the higher quality evidence could then be used to inform guidelines on the use of antimalarial drugs, which based on this more complete, accurate and precise information are likely to be of higher quality themselves, and lead to better outcomes for more patients. In order to assess any impact on patient outcomes, further studies would be required (Ferrante di Ruffano et al., 2012).

Objectives

The objectives of this review are, for clinical blood samples taken from symptomatic or asymptomatic patients known or suspected to carry *Plasmodium falciparum* parasites, to:

- Obtain per study estimates of the sensitivity and specificity of each molecular method for determining the genotype of each drug resistance marker.
- Obtain summary estimates of the sensitivity and specificity of each molecular method for determining the genotype of each drug resistance marker.
- Use statistical methods to compare estimates of sensitivity and specificity of different methods to identify the most sensitive and specific methods.
- Narratively synthesise and compare estimates of sensitivity and specificity to identify the most sensitive and specific methods when statistical synthesis and comparison are not appropriate.

The index and reference tests include any molecular methods for determining the genotype of at least one of the molecular markers selected in this review. Markers were chosen when:

- an individual SNP, or other SNPs in the same gene have been shown to be associated with treatment failure.
- *in vitro* experiments have shown a phenotype of resistance
- emerging evidence shows that markers are likely to be of interest in future surveillance.

Methods

This protocol was prepared following the PRISMA-P and PRISMA guidance, and with reference to AMSTAR and STARD guidance (Moher et al., 2009, Moher et al., 2015, Shamseer et al., 2015, Bossuyt et al., 2015, Shea et al., 2009, Adams et al., 2013).

Eligibility criteria

We will select studies according to the criteria below:

Study design

We will include only diagnostic accuracy studies. We will include case-control studies in the review but may exclude them from the meta-analysis (Whiting et al., 2013).

Participants

We will include studies examining any number of samples, taken from patients or asymptomatic patients when there is a suspicion that they may be carrying *Plasmodium* parasites. We will exclude data generated using lab-adapted *Plasmodium* strains and 'spiked' samples.

Index and Reference tests

One of the major challenges in this review is the choice of a reference standard. The gold standard for genotyping antimalarial drug resistance markers may be next generation sequencing (NGS), but this has not been established by diagnostic test accuracy studies or a systematic review (Fox et al., 2014).

Due to the cost and resource requirements of sequencing it is unlikely that many research groups, particularly those in low and middle-income countries (LMIC), will have access to the facilities they need to use sequencing as the reference standard in their diagnostic accuracy studies. It is also unlikely that many groups who may want to use the findings of this review to inform their choice of molecular method for antimalarial drug resistance genotyping will have access to sequencing.

In order to include studies from LMIC, to include studies more likely to have greater clinical applicability, to include a greater range of tests, and to produce findings useful to as many research

groups as possible, we will include as index and reference tests any molecular methods for determining the presence or the genotype of at least one molecular marker associated with antimalarial drug resistance.

An exhaustive list of tests is not known in advance. We will include all studies comparing at least two methods. Where polymerase chain reaction (PCR) has been carried out to determine presence of *Plasmodium falciparum* we will use this as a reference test to consider sensitivity within and between different studies.

Target condition

The target condition is the genotype, haplotype or copy number of a molecular marker (or markers) of antimalarial drug resistance, listed in Table 29. The quality and type of evidence associating specific markers with antimalarial drug resistance varies, we have attempted to include those markers with the strongest clinical evidence and markers which are linked to resistance to commonly used antimalarial drugs. An update to this review would allow the expansion of the review to include more markers.

We will include studies that examine at least one molecular marker listed in Table 29. We will seek unpublished data for studies that do not provide sufficient data to construct a 2x2 table of test accuracy. Only if results of the target condition can be obtained will the studies be eligible for inclusion (Bossuyt et al., 2015).

Table 29. Selected molecular markers of antimalarial resistance

gene	amino acid	wildtype	mutant	associated with sensitivity or resistance to:
pfcrt (Djimde et al., 2001, Beshir et al., 2010, Picot et al., 2009, Baraka et al., 2015, Otienoburu et al., 2016, Some et al., 2010)	72	C	S	amodiaquine, chloroquine, lumefantrine, piperaquine
	74	M	I	
	75	N	E	
	76	K	T	
plasmodium falciparum cytochrome b (pfcytb) (Musset et al., 2006, Sutherland et al., 2008)	268	Y	N/S/C	atovaquone
pfdhfr (Gadalla et al., 2013, Picot et al., 2009, Warsame et al., 2015)	16	A	V	pyrimethamine
	51	N	I	
	59	C	N	
	108	S	D	
	164	I	L	
pfdhps (Brooks et al., 1994, Reeder et al., 1996, Triglia et al., 1997, Gregson and Plowe, 2005, Menard et al., 2006, Picot et al., 2009, Gadalla et al., 2013, Warsame et al., 2015)	436	S	A/F	sulphadoxine
	437	A	G	
	540	L	E	
	581	A	G	
	613	T	S	
pfk13 (Amaratunga et al., 2016, Phyo et al., 2016, WHO, 2016c, WHO, 2016a)	458	N	Y	artemisinins
	493	Y	H	
	539	R	T	
	543	I	T	
	561	R	H	
	580	C	Y	
Plasmodium falciparum multi-drug resistance 1 (pfmdr1) (Djimde et al., 2001, Picot et al., 2009, Some et al., 2010, Otienoburu et al., 2016, Phyo et al., 2016, Sondo et al., 2016)	86	N	Y	amodiaquine, chloroquine, lumefantrine, mefloquine

Timing

We will exclude studies reported before 1983, when PCR was first developed (Saiki et al., 1985).

Setting

We will include studies from any setting. Where studies examine patients or samples in or from areas where *Plasmodium falciparum* is responsible for <90% of confirmed cases, only studies using a PCR-based method of speciation will be included.

Language

We will not restrict studies by language of publication (Juni et al., 2002).

Publication status

We will include only studies for which there are published reports. Of those published studies, we will seek unpublished results when all other eligibility criteria are met. When results are not recovered, studies will be listed but will not be included in the quantitative or narrative syntheses (Hopewell et al., 2007).

Information sources

We will search Medical Literature Analysis and Retrieval System Online (MEDLINE) via the PubMed interface, Excerpta Medica database (EMBASE) via the OVID interface, BioSciences Information Service of Biological Abstracts (BIOSIS), and Science Citation Index via Web of Knowledge from inception (Hartling et al., 2016).

We will check reference lists of eligible studies identified through the search. We will circulate a list of the eligible reports to authors of included reports and to experts in malaria diagnostics and drug resistance that we have identified.

Search strategy

We have developed specific search strategies following guidance from Higgins and Green, 2008 (Higgins and Green, 2008), Centre for Reviews and Dissemination, 2009 (CRD, 2009), and de Vet et al. 2008 (de Vet, 2008), and will check the strategies with an information specialist. We will make an initial search and a second search later in the review to check for new studies. We will utilise literature

search strategies using medical subject headings (MeSH) and text words related to malaria, drug resistance, genes of interest, and molecular methods. No filters will be used (Ritchie et al., 2007, Rutjes et al., 2007). Often two sets of terms are used to identify studies for reviews of diagnostic test accuracy: terms to identify the index test and terms to identify the target condition. As this review is not restricted to a particular index test, or set of index tests, and a comprehensive list of tests is not known, it would not be possible to use the first set of terms. Instead, we will use terms to identify the target condition. A piloted MEDLINE search strategy is included in Figure 9. We have adapted the MEDLINE search strategy to the syntax and medical subject headings of the other databases.

Figure 9: Piloted MEDLINE search

Patients	"plasmodium falciparum"[MeSH Terms] OR "plasmodium falciparum"[Text Word]
Index Test	-
Reference Test	-
Target Condition	AND
	"chloroquine"[MeSH Terms] OR “chloroquine”[Text Word] OR “CQ”[Text Word] OR “crt”[Text Word] OR “pfcrt”[Text Word] AND resist* [Text Word]
	OR
	"multidrug resistance protein 1"[Text Word] OR “pfmdr1”[Text Word] OR “mdr1”[Text Word] AND resist* [Text Word]
	OR
	"tetrahydrofolate dehydrogenase"[MeSH Terms] OR “pfdhps”[Text Word] OR “dhps”[Text Word] OR “dihydrofolate reductase”[Text Word] AND resist* [Text Word]
	OR
	"dihydropteroate synthase"[MeSH Terms] OR “dihydropteroate synthase”[Text Word] OR “pfdhfr”[Text Word] OR “dhfr”[Text Word] AND resist* [Text Word]
	OR
	“pfk13” [Text Word] OR “Kelch 13”[Text Word] OR “K13”[Text Word] AND resist* [Text Word]
	OR
	"cytochromes b"[MeSH Terms] OR “pfcytb”[Text Word] OR "cytochrome b"[Text Word] OR “cyt-b”[Text Word] OR “cytb”[Text Word] AND resist* [Text Word]

Data management

We will upload literature search results to EndNote 7.7.1 software.

Selection process

We will assess titles, or titles and abstracts of search results using a piloted screening form based on our inclusion and exclusion criteria; the screening tool is available in Figure 10. We will obtain full text of reports for any potentially eligible studies or when there is any uncertainty. We will use the same screening form to assess full-text reports. We will seek additional information from study authors where necessary to resolve questions about eligibility.

Figure 10: Piloted screening tool

Review Author Details	Date screened by Reviewer 1	
	Date screened by Reviewer 2	
	Report screened	
Report Details	Study ID (Trial Registration)	
	Report ID (PMID)	
	Author List	
	Date Published	
	Title	
Inclusion Criteria	Diagnostic accuracy study design, including case-control	
	Examining clinical blood samples taken from patients or asymptomatic patients when there is a knowledge or suspicion that they may be carrying *Plasmodium* parasites	
	Patients or samples in or from countries where *Plasmodium falciparum* is responsible for >90% of confirmed cases with or without speciation	
	Patients or samples in or from countries where *Plasmodium falciparum* is responsible for <90% of confirmed cases with speciation	
	Examining at least one of the antimalarial resistance markers:	
	o *pfcrt* (72, 74, 75, 76)	
	o *pfmdr1* (86, 184, 1034, 1042, copy number)	
	o *pfcytb* (268)	
	o *pfdhps* (436, 437, 540, 581, 613)	
	o *pfdhfr* (16, 51, 59, 108, 164)	
	o *pfk13* (458, 493, 539, 543, 561, 580)	
	Comparing any two or more molecular methods for detecting an antimalarial resistance marker	
	Providing sufficient data to construct a 2x2 table of test accuracy	
Exclusion criteria	Published before 1983	
	Including lab-adapted *Plasmodium* strains and 'spiked' samples	
	No published report	

We will use report and study details to identify multiple reports of the same study or samples; we will compare any potential duplicate reports in full. At any stage of the review if we find multiple reports of the same study, all but one report will be excluded. A second reviewer will independently screen 10% of search results and all reports deemed eligible by the first reviewer (Edwards et al., 2002). We will resolve disagreements through discussion. We will record at least one reason that reports or studies are excluded. Review authors will not be blind to the journal titles, study authors, or institution.

Data collection process

We will extract data all eligible studies using piloted standardised data extraction forms; the data extraction form is available in Figure 11.

Within published studies, we will seek unpublished results from study investigators and report authors. We will use email including a reply form with pre-specified data items (Young and Hopewell, 2011). If data cannot be obtained, we will exclude studies from the analysis and list them in the appendices. A second reviewer will independently extract data (Buscemi et al., 2006). Discrepancies will be resolved by discussion.

Figure 11: Piloted data extraction tool

Review Author Details	Date extracted by Reviewer 1	
	Date extracted by Reviewer 2	
	Request further details from authors?	
Report Details	Study ID (Trial Registration)	
	Report ID (PMID)	
	Author List	
	Date Published	
	Title	
Study	Country of study	
	Date of study	
	Type of laboratory	
Patients	Country of patient screening or diagnosis	
	Date of patient screening or diagnosis	
	Screening / active case finding	
	Diagnosis	
	Eligibility criteria	
	Sex	
	Age	
	Concomitant conditions/infections	
	Sampling method	
	Number of patients	
Samples	Country of *Plasmodium* infection	
	Date of *Plasmodium* infection	
	Source of blood (venous, 100ul, coagulant)	
	Parasitaemia	
	Speciation	
	Multiplicity of infection	
	Storage	
	Number of samples	
	Sampling method	
Index Test	DNA extraction	
	Genotyping conduct	
	Genotyping interpretation	
Reference Test	DNA extraction	
	Genotyping conduct	
	Genotyping interpretation	
Target condition	Genes and SNPs	
Flow and timing	Uninterpretable / indeterminate / missing results	
Blinding	Blinding of participants, clinicians, outcome observers or analysts	
Comparative	Interval between index and reference tests	
COI	Funding and COI	
Other	Notes	

Data items

We will extract report and publication details. We will extract study identifiers and characteristics including design, location and time, patient and sample characteristics, molecular methods used and markers examined, flow and timing of patients and samples through testing. We will extract details on potential sources of bias for assessment using the QUADAS-2 (Quality Assessment of Diagnostic Accuracy Studies) checklist and other items recommended by STARD (Standards for Reporting of Diagnostic Accuracy) (Whiting et al., 2011, Bossuyt et al., 2015). We will extract all results relating to test accuracy. A full list of data items is available in Figure 11.

Where haplotypes are reported, individual genotypes will be extracted if possible. Sensitivity and specificity will be calculated from whatever test accuracy outcomes are provided. If data cannot be obtained in a numeric format, it will be translated from graphically presented data using im2graph 1.2.

Outcomes and prioritisation

Primary outcomes

The primary outcomes will be:

- The sensitivity and specificity from each study, of each molecular method for determining the genotype of any drug resistance markers listed in Table 29. Selected molecular markers of antimalarial resistance.
- A summary estimate of sensitivity and specificity of different methods from studies judged to be sufficiently similar in design and clinical characteristics.
- A comparison of sensitivity and specificity of different methods from studies judged to be sufficiently similar in design and clinical characteristics.

Risk of bias in individual studies

We will assess risk of bias using the piloted adapted QUADAS-2 tool in Figure 12. For each domain two reviewers will independently make an assessment of the risk of bias from the extracted information, and rate the risk of bias as “low”, “high”, or “unclear”. If insufficient data is available in reports or obtained as planned, the risk of bias will be rated as “unclear”. Disagreements will be resolved by discussion. In addition, we will consider other sources of bias (Hirji, 2009, Shinkins et al., 2013, Whiting et al., 2013). We will create a graphical representation of risk of bias within included studies using RevMan 5.3. We will describe how the results of the assessment contribute to the overall findings of the review.

Figure 12: Piloted modified QUADAS-2 tool

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	
	Was a case-control design avoided? Was a range of parasitaemias used?	
	Did the study avoid inappropriate exclusions?	
	Risk of bias: Could the selection of patients have introduced bias?	
DOMAIN 2: Index Test	Applicability: Are there concerns that the included patients and setting do not match the review question?	
	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	
DOMAIN 3: Reference Standard	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	
	Is the reference standard likely to correctly classify the target condition?	
	Were the reference standard results interpreted without knowledge of the results of the index tests?	
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	
DOMAIN 4: Flow and Timing	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	
	Was there an appropriate interval between index test and reference standard (between sample taken)?	
	Did all patients receive the same reference standard?	
	Were all patients included in the analysis?	
	Risk of Bias: Could the patient flow have introduced bias?	

Data Synthesis

We will perform analyses using RevMan 5.3 and Stata 14, and display key characteristics in tables. For each study we will determine sensitivity and specificity with 95% confidence intervals (CIs). Where studies are sufficiently similar in design, setting, patient and sample characteristics, and test conduct, we will conduct meta-analyses using a random-effects model to generate summary estimates of sensitivity and specificity. If we carry out a meta-analysis we will use a meta-regression to examine heterogeneity. We will carry out a sensitivity analysis to examine any assumptions that combining different studies was justified. We will use hierarchical logistic regression to compare estimates of

sensitivity and specificity for different tests. We will calculate correlation coefficients to test relatedness of sensitivity and specificity (Kirkwood et al., 2003).

Where studies are heterogeneous in design, setting, patient and sample characteristics, or test conduct, we will not carry out a meta-analysis, and will synthesise the data through narrative, following guidance previously developed (Popay, 2006).

We will not carry out a formal assessment of publication bias of studies included in this review; techniques such as funnel plots and regression tests have not been found to be useful for identifying publication bias of diagnostic test accuracy studies (Macaskill, 2010).

Confidence in cumulative estimate

We will assess the quality of evidence using Grading of Recommendations Assessment, Development and Evaluation (GRADE) methodology (Schunemann et al., 2008, Schunemann et al., 2016). We will consider additional domains where appropriate. We will designate quality as high (further research is very unlikely to change our confidence in the estimate of effect), moderate (further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate), low (further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate), or very low (very uncertain about the estimate of effect).

This review will help to identify sub-optimal, and more sensitive and specific molecular methods for antimalarial marker detection. By utilising the more accurate methods we can improve the quality of evidence generated by surveillance. Higher quality evidence generated by more accurate and detailed surveillance could be used to inform guidelines on the use of antimalarial drugs, leading to better outcomes for more patients.

Table 30. PRISMA-P 2015 Checklist

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
ADMINISTRATIVE INFORMATION					
Title					
Identification	1a	Identify the report as a protocol of a systematic review	☒	☐	115
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	☐	☒	na
Registration	2	If registered, provide the name of the registry (e.g., PROSPERO) and registration number in the Abstract	☒	☐	115
Authors					
Contact	3a	Provide name, institutional affiliation, and e-mail address of all protocol authors; provide physical mailing address of corresponding author	☐	☒	na
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	☐	☒	na
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	☒	☐	115
Support					
Sources	5a	Indicate sources of financial or other support for the review	☒	☐	115
Sponsor	5b	Provide name for the review funder and/or sponsor	☐	☒	na
Role of sponsor/funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	☐	☒	na
INTRODUCTION					
Rationale	6	Describe the rationale for the review in the context of what is already known	☒	☐	118-121
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	☒	☐	122
METHODS					

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
Eligibility criteria	8	Specify the study characteristics (e.g., PICO, study design, setting, time frame) and report characteristics (e.g., years considered, language, publication status) to be used as criteria for eligibility for the review	☒	☐	123-126
Information sources	9	Describe all intended information sources (e.g., electronic databases, contact with study authors, trial registers, or other grey literature sources) with planned dates of coverage	☒	☐	126-127
Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	☒	☐	127
STUDY RECORDS					
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	☒	☐	127, 129
Selection process	11b	State the process that will be used for selecting studies (e.g., two independent reviewers) through each phase of the review (i.e., screening, eligibility, and inclusion in meta-analysis)	☒	☐	128-129
Data collection process	11c	Describe planned method of extracting data from reports (e.g., piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	☒	☐	129-131
Data items	12	List and define all variables for which data will be sought (e.g., PICO items, funding sources), any pre-planned data assumptions and simplifications	☒	☐	130-131
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	☒	☐	132
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	☒	☐	132-133
DATA					
Synthesis	15a	Describe criteria under which study data will be quantitatively synthesized	☒	☐	133

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data, and methods of combining data from studies, including any planned exploration of consistency (e.g., I^2, Kendall's tau)	☒	☐	133-134
	15c	Describe any proposed additional analyses (e.g., sensitivity or subgroup analyses, meta-regression)	☒	☐	134
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	☒	☐	134
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (e.g., publication bias across studies, selective reporting within studies)	☒	☐	134
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (e.g., GRADE)	☒	☐	134

Characteristics of studies

Table 31. Characteristics of Durand et al. (2000)

Study	Country of study	France
	Date of study	Not described
	Type of laboratory	Hospital Parasitology Laboratory/ National Reference Centre for the Chemosensitivity of Malaria
Patients	Country of patient screening or diagnosis	France
	Date of patient screening or diagnosis	1998-1999
	Screening / symptomatic diagnosis	Symptomatic diagnosis
	Diagnosis	Not described
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Not described
	Number of patients	Not described
Samples	Country of Plasmodium infection	Africa
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Fresh venous blood
	Parasitaemia	Not described
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	Not described
	Number of samples	100
	Sampling method	Not described
Index Test	DNA extraction	Ethanol precipitation
	Genotyping conduct	PCR with molecular beacons: Beacons labelled with fluorophores specific for alleles used in PCR. Read with Fluostar 403 spectrofluorometer.
	Genotyping interpretation	Biolise 2.01 software used to analyse. Determination of thresholds previously done on known samples of a limited series. Results were obtained from simultaneous assays based on triplicate determinations.
Reference Test	DNA extraction	Ethanol precipitation
	Genotyping conduct	PCR-RFLP: PCR followed by digestion by restriction enzymes. Products separated by gel electrophoresis.
	Genotyping interpretation	Bands on gel visualised.
Target condition	Genes and SNPs	*pfdhfr* (108)
Flow and timing	Uninterpretable / indeterminate / missing results	Not described
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Reference test determined while blind to index test results
Comparative	Interval between index and reference tests	Index test before reference test, interval between tests not described
Funding and COI		1. French Ministry of Health 2. Zeneca Pharma 3. Agence Universitaire de la Francophonie 4. WHO/TDR Research Training grant
Notes		

Table 32. Characteristics of Durand et al. (2002)

Study	Country of study	France
	Date of study	2000-2002
	Type of laboratory	Hospital Parasitology Laboratory / National Reference Centre for the Chemosensitivity of Malaria
Patients	Country of patient screening or diagnosis	France
	Date of patient screening or diagnosis	2000-2001
	Screening / symptomatic diagnosis	Symptomatic diagnosis
	Diagnosis	Not described
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Not described
	Number of patients	Not described
Samples	Country of Plasmodium infection	Mostly African countries
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	venous blood
	Parasitaemia	80-7100 trophozoites/µl
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	Not described
	Number of samples	130
	Sampling method	Not described
Index Test	DNA extraction	Ethanol precipitation
	Genotyping conduct	PCR with molecular beacons: Beacons labelled with fluorophores specific for alleles used in PCR, read with Fluostar 403 spectrofluorometer
	Genotyping interpretation	Determination of thresholds previously done on known samples of a limited series.
Reference Test	DNA extraction	Ethanol precipitation
	Genotyping conduct	PCR-RFLP: PCR followed by digestion by restriction enzymes, products separated by gel electrophoresis
	Genotyping interpretation	Bands on gel visualised
Target condition	Genes and SNPs	*pfcrt* (76)
Flow and timing	Uninterpretable / indeterminate / missing results	Not described
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Reference test determined while blind to index test results
Comparative	Interval between index and reference tests	Index test before reference test, interval between tests not described
Funding and COI		Not described
Notes		

Table 33. Characteristics of Kengne et al. (2003)

Study	Country of study	Burkina Faso, France
	Date of study	Not described
	Type of laboratory	Parasitology Laboratory / National Research Centres
Patients	Country of patient screening or diagnosis	Burkina Faso or France
	Date of patient screening or diagnosis	Not described
	Screening / symptomatic diagnosis	Not described
	Diagnosis	Not described
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Not described
	Number of patients	Not described
Samples	Country of Plasmodium infection	Burkina Faso
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Not described
	Parasitaemia	Not described
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	50ul spotted in triplicate onto Whatman filter paper, 2ml parasites pelleted and stored at -20C
	Number of samples	56
	Sampling method	Not described
Index Test	DNA extraction	Chelex extraction
	Genotyping conduct	ASPCR: PCR followed by electrophoresis and UV visualisation, in some cases DNA quantity adjusted and retested.
	Genotyping interpretation	Not described
Reference Test	DNA extraction	Chelex extraction
	Genotyping conduct	PCR-RFLP: PCR followed by digestion by restriction enzymes. Products separated by gel electrophoresis.
	Genotyping interpretation	Not described
Target condition	Genes and SNPs	*pfdhps* (108)
Flow and timing	Uninterpretable / indeterminate / missing results	Not described
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. AUPELF-UREF
Notes		

Table 34. Characteristics of Alker et al. (2004)

Study	Country of study	USA
	Date of study	Not described
	Type of laboratory	University Research Laboratories, University Sequencing Core
Patients	Country of patient screening or diagnosis	Malawi
	Date of patient screening or diagnosis	December 2000 - June 2002
	Screening / symptomatic diagnosis	Screening
	Diagnosis	Peripheral and placental thick blood films
	Eligibility criteria	Not described
	Sex	Female
	Age	Not described
	Concomitant conditions/infections	24 late third trimester of pregnancy 21 HIV+ve 1 HIV-ve 2 HIV+ve, aparasitaemic
	Sampling method	Unclear method of sampling 24 participants from larger cohort study of 480 participants
	Number of patients	24
Samples	Country of Plasmodium infection	Malawi
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Peripheral venous blood
	Parasitaemia	Not described
	Speciation	Peripheral and placental thick blood films examined by two trained microscopists.
	Multiplicity of infection	Not described
	Storage	Plasma and packed-cell fractions stored at 80°C. Thawed, and a few drops were transferred onto filter paper and shipped.
	Number of samples	24
	Sampling method	5 samples chosen to receive reference test based on result of index test, case control design.
Index Test	DNA extraction	QIAamp DNA minikit
	Genotyping conduct	Real-time PCR: Fluorophore labelled, with quencher and minor groove binder linked probes. PCR in duplicate on ABI Prism 7000 sequence detection system. Data manipulated (baseline subtracted, replicates averaged) in Microsoft Excel.
	Genotyping interpretation	Genotype determined by cycle threshold (CT) of the two probes for each sample.
Reference Test	DNA extraction	QIAamp DNA minikit
	Genotyping conduct	Sequencing: same primers as used in index test, reactions run on ABI Prism 7000 sequence detection system. Dissociation curves examined and amplicon purified using CentriSpin-10 columns. Sequencing performed using ABI Prism BigDye terminator cycle sequencing ready reaction kit.
	Genotyping interpretation	Not described
Target condition	Genes and SNPs	DHFR (51, 59, 108) DHPS (437, 436, 540, 581, 613)
Flow and timing	Uninterpretable / indeterminate / missing results	Index test results not described for 19/24 samples
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Index test before reference test, no interval between samples, interval between tests not described
Funding and COI		1. NIH grants AI45426 and AI49084 2. NIH-FIC grant 5 D43 TW00908 3. ASPH/CDC cooperative agreement S1935-21/22 4. Center for AIDS research at the University of North Carolina 5. UNC Chapel Hill
Notes		

Table 35. Characteristics of Wilson et al. (2005b)

Study	Country of study	USA, DRC, Malawi
	Date of study	Not described
	Type of laboratory	Not described
Patients	Country of patient screening or diagnosis	DRC Malawi
	Date of patient screening or diagnosis	DRC - June-September 2002 Malawi - January-March 2004
	Screening / symptomatic diagnosis	DRC - not described Malawi – screening
	Diagnosis	Not described
	Eligibility criteria	DRC - *P. falciparum* malaria Malawi – malaria
	Sex	DRC - not described Malawi – female
	Age	DRC - average 28 months, range 6-58 Malawi - not described
	Concomitant conditions/infections	DRC - not described Malawi - third trimester pregnancy
	Sampling method	Not described
	Number of patients	Not described
Samples	Country of Plasmodium infection	DRC, Malawi
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	DRC - not described Malawi - EDTA anticoagulant
	Parasitaemia	DRC - average 1600 asexual parasites /200 WBC, range 36-7200 Malawi - average 1200 parasites /200 WBC, range 2-4000
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	DRC - dried blood spots, blood applied to isocode stix and dried prior to desiccated storage Malawi - -80°C
	Number of samples	DRC 56, Malawi 12
	Sampling method	Not described
Index Test	DNA extraction	DRC - Isocode Stix DNA elution protocol Malawi - Minicolumn purification (Qiagen)
	Genotyping conduct	RT-PCR with MGB: performed with an ABI Prism™ 7000 Sequence Detection System
	Genotyping interpretation	The commonly used Ct value (threshold cycle) was calculated as the intersection of a line drawn through the baseline and a line drawn through the riseline. PCR fluorograms having a clearly defined Ct and a linear riseline slope above the cut-off minimum found using mixed reference standards were scored as positive.
Reference Test	DNA extraction	DRC - Isocode Stix DNA elution protocol Malawi - Minicolumn purification (Qiagen)
	Genotyping conduct	ASPCR: DNA polymerisation was performed in a PTC-200 Thermocycler and product from the second round of PCR electrophoresed on agarose gel.
	Genotyping interpretation	The presence of the expected 200 bp DNA fragment was diagnostic of either the wild-type or mutant genotype.
Target condition	Genes and SNPs	*pfcrt* (76)
Flow and timing	Uninterpretable / indeterminate / missing results	Not described
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. grant S1935-21/21 from the CDC 2. grants RO1 AI 49084 and U01 HD043475-01 from the NIH
Notes		Results for 2x2 table missing for sequencing as reference test

Table 36. Characteristics of Farcas et al. (2006)

Study	Country of study	Canada
	Date of study	Not described
	Type of laboratory	Hospital Tropical Disease Unit, University Centre for Molecular Medicine
Patients	Country of patient screening or diagnosis	Canada
	Date of patient screening or diagnosis	July 1999 to March 2003
	Screening / symptomatic diagnosis	Symptomatic diagnosis
	Diagnosis	Microscopy and PCR
	Eligibility criteria	Fever, or a history of fever within 48 h, and travel to a malaria-endemic area.
	Sex	8:5
	Age	mean 31 ± 1.6 years (SD), range 4 months–73 years
	Concomitant conditions/infections	Not described
	Sampling method	Consecutive patients who presented or were referred to the Tropical Disease Unit of the University Health Network–Toronto General Hospital, and, during the first 3 months of the study, patients who repeatedly had negative blood film results, and, consecutive patients with Plasmodium *vivax*, *malariae*, or *ovale* were included.
	Number of patients	200
Samples	Country of Plasmodium infection	Africa (78%), Indian subcontinent (10%), South and Central America (5%), Oceania (4%), Southeast Asia (3%)
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Whole blood
	Parasitaemia	range 20-500,000 parasites/ml, 8% of samples <100 parasites/ml
	Speciation	microscopy and PCR
	Multiplicity of infection	Not described
	Storage	Not described
	Number of samples	200
	Sampling method	Consecutive
Index Test	DNA extraction	Minicolumn purification from 200ul whole blood (Qiagen)
	Genotyping conduct	RT-PCR: PCR using fluorescent probes, LightCycler platform
	Genotyping interpretation	Not described
Reference Test	DNA extraction	Minicolumn purification from 200ul whole blood (Qiagen)
	Genotyping conduct	PCR-RFLP: PCR followed by digestion by restriction enzymes. Products separated by gel electrophoresis.
	Genotyping interpretation	Bands on gel visualised
Target condition	Genes and SNPs	*pfcrt* (76)
Flow and timing	Uninterpretable / indeterminate / missing results	Not described
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Performed independently and in a blinded manner
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. Canadian Institutes of Health Research (MT-13701) 2. Genome Canada 3. Artus employee
Notes		

Table 37. Characteristics of Juliano et al. (2007)

Study	Country of study	Malawi, USA
	Date of study	Not described
	Type of laboratory	Not described
Patients	Country of patient screening or diagnosis	Malawi
	Date of patient screening or diagnosis	Not described
	Screening / symptomatic diagnosis	Screening
	Diagnosis	Blantyre - histopathological examination of placental tissue Mpemba and Madziabango - microscopy
	Eligibility criteria	Not described
	Sex	Women
	Age	21.9 (15–35) years
	Concomitant conditions/infections	Pregnant 87/87, Anaemia 64/87, HIV status 38/58
	Sampling method	Blantyre - women admitted in the antenatal ward were screened for eligibility Mpenmba and Madziabango - randomised, open-label study to assess complexity of infection and treatment efficacy
	Number of patients	87
Samples	Country of Plasmodium infection	Malawi
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	peripheral blood
	Parasitaemia	243.6 parasites / 200 WBC (4–2,202)
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	filter paper blood spots
	Number of samples	87
	Sampling method	Not described
Index Test	DNA extraction	Not described
	Genotyping conduct	PCR with isotopic multiple site-specific heteroduplex tracking assay (MSS-HTA): annealing reaction and electrophoresis, gels dried onto filter paper and exposed to X-ray film, duplicate
	Genotyping interpretation	Phosphorimager screen, band intensities quantified by GE Storm 860 Phosphorimager and ImageQuant version 5.2 software
Reference Test	DNA extraction	Not described
	Genotyping conduct	allele-restricted PCR (ARPCR): same as ASPCR
	Genotyping interpretation	Not described
Target condition	Genes and SNPs	*pfcrt* (72-76)
Flow and timing	Uninterpretable / indeterminate / missing results	Blantyre - received both MSS-HTA and ARPCR Mpenmba and Madziabango - received MSS-HTA, ARPCR was performed on all samples positive by HTA and on a random selection of 20 MSS-HTA–negative samples
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. American Society of Public Health/CDC S193521/21 2. NIAID/NIH AI49804 3. NIH training grant DHHS/NIH/NIAID 5 T32 AI 07001-28
Notes		Patients described further in PMID: 1. 17116685 2. 16954728

Table 38. Characteristics of Juliano et al. (2008)

Study	Country of study	Malawi, USA
	Date of study	Not described
	Type of laboratory	Not described
Patients	Country of patient screening or diagnosis	Malawi
	Date of patient screening or diagnosis	Not described
	Screening / symptomatic diagnosis	Screening
	Diagnosis	Blantyre - histopathological examination of placental tissue Mpemba and Madziabango - microscopy
	Eligibility criteria	Not described
	Sex	women
	Age	Not described
	Concomitant conditions/infections	Pregnancy
	Sampling method	Blantyre - women admitted in the antenatal ward were screened for eligibility Mpenmba and Madziabango - randomised, open-label study to assess complexity of infection and treatment efficacy
	Number of patients	85 and 62
Samples	Country of Plasmodium infection	Malawi
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Peripheral blood
	Parasitaemia	Not described
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	Filter paper blood spots or frozen blood
	Number of samples	85 and 62
	Sampling method	Not described
Index Test	DNA extraction	Minicolumn purification (Qiagen)
	Genotyping conduct	PCR with isotopic multiple site-specific heteroduplex tracking assay (MSS-HTA): annealing reaction and electrophoresis, gels dried onto filter paper and exposed to X-ray film, duplicate
	Genotyping interpretation	Phosphorimager screen, band intensities quantified by GE Storm 860 Phosphorimager and ImageQuant version 5.2 software
Reference Test	DNA extraction	Minicolumn purification (Qiagen)
	Genotyping conduct	Real-time PCR: Fluorophore labelled, with quencher and minor groove binder linked probes. PCR in duplicate on ABI Prism 7000 sequence detection system. Data manipulated (baseline subtracted, replicates averaged) in Microsoft Excel.
	Genotyping interpretation	Genotype determined by cycle threshold (CT) of the two probes for each sample.
Target condition	Genes and SNPs	*pfdhfr* (164)
Flow and timing	Uninterpretable / indeterminate / missing results	Results missing for some samples, not described.
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Reference test carried out before index test for 85 samples, and index test before reference test for 62 samples.
Funding and COI		1. ASPH/CDC S1935-21/21 2. NIAID/NIH AI49804 3. Infectious Disease Pathogenesis Research Training grant (DHHS/NIH/NIAID 5 T32 AI07151-29) 4. 2007 IDSA ERF/NFID Merle A. Sande/Pfizer Fellowship in International Infectious Diseases.
Notes		Patient characteristics described further in PubMed: 1. 16127071 2. 15096809

Table 39. Characteristics of Mens et al. (2008)

Study	Country of study	Belgium, France, the Netherlands
	Date of study	Not described
	Type of laboratory	Not described
Patients	Country of patient screening or diagnosis	The Democratic Republic of Congo
	Date of patient screening or diagnosis	September 2003 to March 2004
	Screening / symptomatic diagnosis	Not described
	Diagnosis	Not described
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Observational study of SP efficacy
	Number of patients	Not described
Samples	Country of Plasmodium infection	The Democratic Republic of Congo
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Finger-prick blood
	Parasitaemia	range: 16 -197,400 parasites / µl
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	3M Whatman filter paper, stored at room temperature in an individual zip-lock polyethylene bag until further DNA extraction
	Number of samples	78
	Sampling method	Not described
Index Test	DNA extraction	Not described
	Genotyping conduct	RT-PCR-MCA/FRET: Real-time PCR with melting curve analysis using fluorescence resonance energy transfer. PCR is followed by melt curve analysis (MCA) and dissociation of the fluorophores. Performed on an iCycler and analysis of the results was done by iCyclerIQ optical system software version 3.0a. All experiments were performed in triplicate.
	Genotyping interpretation	Not described
Reference Test	DNA extraction	Not described
	Genotyping conduct	PCR-RFLP: PCR followed by restriction digestion. Products visualised by a UV light transilluminator agarose gel electrophoresis.
	Genotyping interpretation	Not described
Target condition	Genes and SNPs	*pfdhps* (540)
Flow and timing	Uninterpretable / indeterminate / missing results	Not described
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. Fund for Scientific Research/Fonds voor Wetenschappelijk Onderzoek (NavorserFWO) 2. Médecins sans Frontières, Belgium
Notes		More details of patients and samples in PMID: 1. 16837723 2. 9603482

Table 40. Characteristics of Andriantsoanirina et al. (2009)

Study	Country of study	France
	Date of study	Not described
	Type of laboratory	Hospital Parasitology Laboratory, University Genomics Laboratory
Patients	Country of patient screening or diagnosis	Madagascar, Comoros Islands
	Date of patient screening or diagnosis	2006-2007
	Screening / symptomatic diagnosis	Symptomatic diagnosis
	Diagnosis	Blood smear / thick blood smear
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	From multi-site randomized clinical trial of CQ, SP AQ and ASAQ efficacy
	Number of patients	Not described
Samples	Country of Plasmodium infection	Madagascar, Comoros Islands
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	venous blood samples, EDTA tubes
	Parasitaemia	Not described
	Speciation	Blood smears
	Multiplicity of infection	Not described
	Storage	+4°C within 48 hours of collection
	Number of samples	177
	Sampling method	Not described
Index Test	DNA extraction	100µl blood, phenol-chloroform extraction
	Genotyping conduct	Real-time PCR: High Resolution Melting, PCR and HRM in duplicate on Rotorgene 6000. Analysis by Rotorgene software.
	Genotyping interpretation	Genotype determined by normalised and difference graphs by Rotorgene software.
Reference Test	DNA extraction	100µl blood, phenol-chloroform extraction
	Genotyping conduct	Sequencing: bands visualised by agarose gel electrophoresis and purified by filtration using Macherey-Nagel plate. Sequencing using ABI Prism BigDye Terminator cycle sequencing ready reaction kit run on a 3730 XL genetic analyser.
	Genotyping interpretation	Electropherograms were visualised and analysed with Seqscape software. Aa sequences were compared using BioEdit Sequence Alignment Editor software.
Target condition	Genes and SNPs	*pfcrt* (76), *pfmdr* (86), *pfdhfr* (51, 59, 108, 164), *pfdhps* (436, 437, 540)
Flow and timing	Uninterpretable / indeterminate / missing results	Same sample used
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. Natixis/Impact Malaria through the Observatoire de la Résistance aux Antipaludiques Project (ORA) 2. Genomics Platform, Pasteur Génopôle, Institut PasteurFunding from the French Government through the FSP/RAI 2001-168 project 4. Global Fund project for Madagascar round 3 (Community Action to Roll Back Malaria, Grant number: MDG-304-G05-M) 5. Institut Pasteur de Madagascar.
Notes		Associated study registered ISRCTN36517335

Table 41. Characteristics of Juliano et al. (2009)

Study	Country of study	Madagascar, Malawi
	Date of study	Not described
	Type of laboratory	Not described
Patients	Country of patient screening or diagnosis	Madagascar, Malawi
	Date of patient screening or diagnosis	Malawi - not described Madagascar - 2002-2004
	Screening / symptomatic diagnosis	Malawi - not described Madagascar - screening
	Diagnosis	Malawi - microscopy Madagascar - not described
	Eligibility criteria	Not described
	Sex	Malawi - female Madagascar - not described
	Age	Malawi - maximum range 15-35 years Madagascar - average 80 months (14-311)
	Concomitant conditions/infections	Malawi - pregnant Madagascar - not described
	Sampling method	Malawi - randomised, open-label study to assess complexity of infection and treatment efficacy Madagascar - from treated with CQ for uncomplicated malaria
	Number of patients	Madagascar - 17 Malawi - not described
Samples	Country of Plasmodium infection	Madagascar, Malawi
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Malawi - peripheral blood Madagascar - not described
	Parasitaemia	Madagascan 0-128,000 trophozoites/ µl Malawi - not described
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	Malawi - filter paper blood spots Madagascar - not described
	Number of samples	Madagascar - 31 Malawi - 24
	Sampling method	Not described

Index Test	DNA extraction	Malawi - Minicolumn purification (Qiagen) Madagascar - not described
	Genotyping conduct	PCR digoxigenin (DIG)-labeled chemiluminescent heteroduplex tracking assay (DIG-HTA): Annealed heteroduplexes were separated on polyacrylamide gel then electroblotted and fixed using a UV crosslinker. Chemiluminescent detection using DIG Luminescent Detection Kit. For autoradiograhic detection, the membrane was exposed to Hyperfilm.
	Genotyping interpretation	Not described
Reference Test (1)	DNA extraction	Malawi - Minicolumn purification (Qiagen) Madagascar - not described
	Genotyping conduct	PCR-RFLP: PCR followed by digestion by restriction enzymes.
	Genotyping interpretation	Not described
Reference Test (2)	DNA extraction	Not described
	Genotyping conduct	PCR with isotopic multiple site-specific heteroduplex tracking assay (MSS-HTA): annealing reaction and electrophoresis, gels dried onto filter paper and exposed to X-ray film.
	Genotyping interpretation	Phosphorimager screen, band intensities quantified by GE Storm 860 Phosphorimager and ImageQuant version 5.2 software
Target condition	Genes and SNPs	*pfcrt* (76)
Flow and timing	Uninterpretable / indeterminate / missing results	PCR-MSS-HTA carried out before PCR-DIG-HTA
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. Medicines for Malaria Venture (MMV) 2. Infectious Disease Pathogenesis Research Training Grant (DHHS/NIH/NIAID 5 T32 AI07151-29) 3. NIAID 1R21AI076785 4. 2007 IDSA ERF/NFID Merle A. Sande/Pfizer Fellowship in International Infectious Diseases 5. Award Number KL2RR025746 from the National Center for Research Resources
Notes		Study designed to test ability to detect minority alleles. Reference test MSS-HTA carried out in Juliano 2007. Extra information on samples in PMID: 1. 18000538 2. 17116685 2. 16954728

Table 42. Characteristics of Cruz et al. (2010)

Study	Country of study	Canada
	Date of study	Not described
	Type of laboratory	Laboratory for Public Health
Patients	Country of patient screening or diagnosis	Travelers and migrants to Canada
	Date of patient screening or diagnosis	Not described
	Screening / symptomatic diagnosis	Symptomatic diagnosis
	Diagnosis	RT-PCR
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Travelers and migrants to Canada
	Number of patients	Not described
Samples	Country of Plasmodium infection	Not described
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Not described
	Parasitaemia	low level, at least 12 had a parasitaemia of less than 0.1% by microscopy
	Speciation	RT-PCR
	Multiplicity of infection	Not described
	Storage	Not described
	Number of samples	46
	Sampling method	Obtained from the Provincial Laboratory for Public Health in Edmonton
Index Test	DNA extraction	Magnetic beads: Precision System Science DNA Magtration system 12GC from 40ul whole blood
	Genotyping conduct	RT-PCR-MCA on Lightcycler platform
	Genotyping interpretation	software
Reference Test	DNA extraction	Magnetic beads: Precision System Science DNA Magtration system 12GC from 40ul whole blood
	Genotyping conduct	Sequencing: Pyrosequencing on a PSQ 96MA (51, 59, 108) and direct sequencing (164), purified PCR products sequenced in both directions (Applied Genomics Centre, University of Alberta) in triplicate
	Genotyping interpretation	Not described
Reference Test	DNA extraction	Magnetic beads: Precision System Science DNA Magtration system 12GC from 40ul whole blood
	Genotyping conduct	RT-PCR: ABI Prism 7000 sequence detection system
	Genotyping interpretation	Not described
Target condition	Genes and SNPs	*pfdpfr* (51, 59, 108, 164)
Flow and timing	Uninterpretable / indeterminate / missing results	Not described
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Blinded reference test
Comparative	Interval between index and reference tests	Not described
Funding and COI		Not described
Notes		

Table 43. Characteristics of Gan and Loh (2010)

Study	Country of study	Singapore
	Date of study	Not described
	Type of laboratory	Population Genetics Laboratory, Diagnostic Laboratory, Defence Science Organisation
Patients	Country of patient screening or diagnosis	Not described
	Date of patient screening or diagnosis	Not described
	Screening / symptomatic diagnosis	Not described
	Diagnosis	Not described
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Not described
	Number of patients	Not described
Samples	Country of Plasmodium infection	Not described
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Not described
	Parasitaemia	Parasitaemia greater than 0.05%
	Speciation	PCR
	Multiplicity of infection	Not described
	Storage	Not described
	Number of samples	53
	Sampling method	Part of a collection from DSO National Laboratories
Index Test	DNA extraction	Minicolumn purification from 200ul whole blood (Qiagen)
	Genotyping conduct	PCR-HRM: PCR conducted on a thermal cycler and melt curves generated using a LightScanner.
	Genotyping interpretation	Not described
Reference Test (1)	DNA extraction	Minicolumn purification from 200ul whole blood (Qiagen)
	Genotyping conduct	RT-PCR: multiplex, real-time PCR (qPCR) in Corbett Rotorgene 3000
	Genotyping interpretation	Samples were considered positive for a particular genotype if a CT value of 35 cycles or fewer was obtained in at least two independent PCR experiments.
Reference Test (2)	DNA extraction	Minicolumn purification from 200ul whole blood (Qiagen)
	Genotyping conduct	PCR-RFLP: nested PCR, incubation with enzyme, electrophoresis in ethidium bromide-stained agarose gel.
	Genotyping interpretation	Not described
Target condition	Genes and SNPs	*pfcrt* (72-76), *pfcytb* (268)
Flow and timing	Uninterpretable / indeterminate / missing results	Not described
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	PCR-RFLP carried out first, interval duration not described
Funding and COI		None declared
Notes		

Table 44. Characteristics of Muhamad et al. (2011)

Study	Country of study	Thailand
	Date of study	2009
	Type of laboratory	Not described
Patients	Country of patient screening or diagnosis	Thailand
	Date of patient screening or diagnosis	Not described
	Screening / symptomatic diagnosis	Diagnosis
	Diagnosis	Not described
	Eligibility criteria	Patients with acute, uncomplicated *P. falciparum*
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Not described
	Number of patients	Not described
Samples	Country of Plasmodium infection	Thailand
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Not described
	Parasitaemia	Not described
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	filter paper (Whatman no. 3)
	Number of samples	96
	Sampling method	Not described
Index Test	DNA extraction	DNA was extracted from dried blood spots by using QIAcubeTM automated sample preparation.
	Genotyping conduct	RT-PCR SYBR Green 1: Samples were analysed in a minimum of 2 independent experiments, with triplicate wells for each experiment. Gene copy number was determined by relative quantification between *pfmdr1* gene and b-actin based on the DDCt method.
	Genotyping interpretation	Not described
Reference Test	DNA extraction	DNA was extracted from dried blood spots by using QIAcubeTM automated sample preparation.
	Genotyping conduct	RT-PCR: with TaqMan RT-quantitative (q)PCR methods with iCycler thermal cycler system. Samples were analysed in a minimum of 2 independent experiments, with triplicate wells for each experiment. Gene copy number was determined by relative quantification between *pfmdr1* gene and b-tubulin genes based on the DDCt method.
	Genotyping interpretation	Not described
Target condition	Genes and SNPs	*pfmdr1* (copy number)
Flow and timing	Uninterpretable / indeterminate / missing results	96 samples tested by index, 63 by reference
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. Commission on Higher Education, Ministry of Education of Thailand 2. National Research University Project (NRU) of Thailand, Office of Higher Education Commission
Notes		

Table 45. Characteristics of Moers et al. (2015)

Study	Country of study	United Kingdom, The Netherlands
	Date of study	Not described
	Type of laboratory	Not described
Patients	Country of patient screening or diagnosis	Kenya, UK
	Date of patient screening or diagnosis	Kenya - 2009 MRL - not described
	Screening / symptomatic diagnosis	Kenya - not described MRL - symptomatic diagnosis
	Diagnosis	Kenya - not described MRL - microscopy and PCR
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Kenya - selected from a clinical trial in Mbita, Kenya MRL - selected from available DNA in the United Kingdom Malaria Reference Laboratory (MRL) archive
	Number of patients	Not described
Samples	Country of Plasmodium infection	Kenya, World-wide
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Kenya - not described MRL - peripheral blood
	Parasitaemia	Kenya - not described MRL - 48 ≥2% of peripheral erythrocytes infected, 92 low parasitaemia, 6 negative by nested PCR. Many of the samples used were sub patent infections undetected by microscopy, which had been confirmed to be parasite positive by PCR.
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	Not described
	Number of samples	Kenya - 17 MRL - 150
	Sampling method	Not described
Index Test	DNA extraction	DNA was isolated by minicolumn purification (Qiagen)
	Genotyping conduct	PCR-PEXT (Primer extension methods) -NALFIA (nucleic acid lateral flow immunoassay) Veriti 96-well thermal cycler, NALFIA strips scanned by a GS-800 calibrated densitometer.
	Genotyping interpretation	Not described
Reference Test	DNA extraction	DNA was isolated by minicolumn purification (Qiagen)
	Genotyping conduct	Sequencing: BigDye® Terminator v3.1 Cycle Sequencing Kit, analysed by ABI 7300. Sequences were viewed and edited in Chromas version 2.31 and aligned in Bioedit sequence analysis software.
	Genotyping interpretation	Not described
Reference Test	DNA extraction	DNA was isolated by minicolumn purification (Qiagen)
	Genotyping conduct	RT-PCR
	Genotyping interpretation	Not described
Target condition	Genes and SNPs	*pfcrt* (76), *pfmdr1* (86, 184, 1246)
Flow and timing	Uninterpretable / indeterminate / missing results	Not described
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Index test conduct and interpretation blinded
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. EU-funded FP7 project MALACTRES grant 201889.
Notes		

Table 46. Characteristics of Sharma et al. (2016)

Study	Country of study	India
	Date of study	Not described
	Type of laboratory	Not described
Patients	Country of patient screening or diagnosis	India
	Date of patient screening or diagnosis	Not described
	Screening / symptomatic diagnosis	Not described
	Diagnosis	Microscopy, PCR
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Collected from malaria-positive patients visiting the primary health centre (PHC) situated in different endemic areas of district Rourkela, Odisha.
	Number of patients	Not described
Samples	Country of Plasmodium infection	India
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Finger pricked blood samples
	Parasitaemia	Not described
	Speciation	PCR
	Multiplicity of infection	Not described
	Storage	Whatman filter paper #3
	Number of samples	55
	Sampling method	Not described
Index Test	DNA extraction	Extracted from dried filter paper blood spots using minicolumn purification (Qiagen)
	Genotyping conduct	ASPCR: primary PCR product was used as the template for eight different allele-specific nested PCRs, amplified PCR products electrophoresed and visualised using UV in the gel documentation system. Samples that were negative for any specific ASPCR were re-genotyped to confirm that the negativity is not due to PCR failure.
	Genotyping interpretation	Not described
Reference Test	DNA extraction	Extracted from dried filter paper blood spots using minicolumn purification (Qiagen)
	Genotyping conduct	Sequencing: PCR products were reamplified by nested PCR electrophoresed and visualised, purified using QIAquick PCR Purification kit. Sequenced using a Big-Dye Terminator kit version 3.1 on both strands of DNA.
	Genotyping interpretation	Individual chromatograms were edited manually and aligned using ClustalW implemented in MEGA version 6. GenBank accession number J03028.1 was used as a reference for the identification of mutations.
Target condition	Genes and SNPs	*pfdhfr* (51, 59, 108, 164)
Flow and timing	Uninterpretable / indeterminate / missing results	None
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. Senior Research Fellowship by University Grants Commission, Government of India 2. National Institute of Malaria Research (NIMR/VBC/2010/162) 3. National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH) grant U19AI089676
Notes		

Table 47. Characteristics of Chahar et al. (2017)

Study	Country of study	India
	Date of study	Not described
	Type of laboratory	National Institute of Malaria Research
Patients	Country of patient screening or diagnosis	India
	Date of patient screening or diagnosis	Not described
	Screening / symptomatic diagnosis	Not described
	Diagnosis	Not described
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Not described
	Number of patients	Not described
Samples	Country of Plasmodium infection	Not described
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Not described
	Parasitaemia	Not described
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	Not described
	Number of samples	50
	Sampling method	Not described
Index Test	DNA extraction	QIAamp DNA Blood Mini/Maxi Kit
	Genotyping conduct	LAMP assay was based on the assessment of calcein colour change reaction, in triplicate and repeated three times.
	Genotyping interpretation	Naked eye visualisation of colour change from light orange to bright yellow for 76T.
Index Test	DNA extraction	QIAamp DNA Blood Mini/Maxi Kit
	Genotyping conduct	LAMP assay was based on the assessment of pattern of band in gel electrophoresis, in triplicate and repeated three times.
	Genotyping interpretation	Trans UV light used to identify gel electrophoresis band patterns.
Reference Test	DNA extraction	QIAamp DNA Blood Mini/Maxi Kit
	Genotyping conduct	Sequencing, through commercially available service
	Genotyping interpretation	Not described
Target condition	Genes and SNPs	*pfcrt* (76)
Flow and timing	Uninterpretable / indeterminate / missing results	Index test, then reference test
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. Indian Council of Medical Research 2. National Institute of Malaria Research
Notes		PCR-RFLP data insufficient for 2x2

Table 48. Characteristics of Pholwat et al. (2017)

Study	Country of study	USA, Uganda, China
	Date of study	Not described
	Type of laboratory	Not described
Patients	Country of patient screening or diagnosis	Thailand, China, Malawi, Uganda
	Date of patient screening or diagnosis	Thailand - 2005 China - 2016 Malawi - 2012 to 2013 Uganda - 2016, 2008 to 2009
	Screening / symptomatic diagnosis	Not described
	Diagnosis	Not described
	Eligibility criteria	Not described
	Sex	Not described
	Age	Not described
	Concomitant conditions/infections	Not described
	Sampling method	Not described
	Number of patients	Not described
Samples	Country of Plasmodium infection	Malawi, Thailand, China, Uganda
	Date of Plasmodium infection	Not described
	Source of blood (venous, 100ul, coagulant)	Not described
	Parasitaemia	Not described
	Speciation	Not described
	Multiplicity of infection	Not described
	Storage	Thailand - dried blood spots China - purified DNA Malawi - purified DNA Uganda - blood pellet or purified DNA
	Number of samples	Thailand - 32 China - 21 Malawi - 28 Uganda - 6
	Sampling method	Not described
Index Test	DNA extraction	DNA was isolated by minicolumn purification (Qiagen)
	Genotyping conduct	TaqMan array card, TAC
	Genotyping interpretation	Not described
Reference Test	DNA extraction	DNA was isolated by minicolumn purification (Qiagen)
	Genotyping conduct	Sequencing: PCR was performed on a CFX96 instrument, purified, submitted to GeneWiz
	Genotyping interpretation	Not described
Target condition	Genes and SNPs	*pfcrt* (72-76), *pfcytb* (268), *pfdhfr* (51, 59, 108, 164), *pfdhps* (436-437, 540, 581, 613), *pfk13* (458, 493, 539, 543, 561, 580), *pfmdr1* (86, 184, 1034, 1042, 1246)
Flow and timing	Uninterpretable / indeterminate / missing results	Reference test carried out on ~50% of samples
Blinding	Blinding of participants, clinicians, outcome observers or analysts	Not described
Comparative	Interval between index and reference tests	Not described
Funding and COI		1. NIH grant K24 AI102972 2. WHO Mekong Malaria Programme WP/10/MVP/005837
Notes		

Methodological quality of studies

Table 49. Methodological quality of Durand et al. (2000)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Yes
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Yes
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Low
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Low
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Low
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Low
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 50. Methodological quality of Durand et al. (2002)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Yes
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Yes
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Low
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Low
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	No
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Low
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Low
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 51. Methodological quality of Kengne et al. (2003)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 52. Methodological quality of Alker et al. (2004)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	No
	Was a case-control design avoided? Was a range of parasitaemias used?	No, and unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Yes
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	No
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	No
	Were all patients included in the analysis?	No
	Risk of Bias: Could the patient flow have introduced bias?	High

Table 53. Methodological quality of Wilson et al. (2005b)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	No, yes
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	No
	Risk of Bias: Could the patient flow have introduced bias?	High

Table 54. Methodological quality of Farcas et al. (2006)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Yes
	Was a case-control design avoided? Was a range of parasitaemias used?	Yes
	Did the study avoid inappropriate exclusions?	Yes
	Risk of bias: Could the selection of patients have introduced bias?	Low
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Low
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Yes
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Low
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Low
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 55. Methodological quality of Juliano et al. (2007)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	No
	Were all patients included in the analysis?	No
	Risk of Bias: Could the patient flow have introduced bias?	High

Table 56. Methodological quality of Juliano et al. (2008)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	No
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	No
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	No
	Risk of Bias: Could the patient flow have introduced bias?	High

Table 57. Methodological quality of Mens et al. (2008)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 58. Methodological quality of Andriantsoanirina et al. (2009)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Yes
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	No
	Risk of Bias: Could the patient flow have introduced bias?	High

Table 59. Methodological quality of Juliano et al. (2009)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 60. Methodological quality of Cruz et al. (2010)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Low
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Low
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 61. Methodological quality of Gan and Loh (2010)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Low
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Low
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 62. Methodological quality of Muhamad et al. (2011)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	No
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 63. Methodological quality of Moers et al. (2015)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Yes
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Low
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Low
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 64. Methodological quality of Sharma et al. (2016)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 65. Methodological quality of Chahar et al. (2017)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	No
	Was a case-control design avoided? Was a range of parasitaemias used?	No, unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	Yes
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

Table 66. Methodological quality of Pholwat et al. (2017)

	Item	Authors' judgement
DOMAIN 1: Patient Selection	Was a consecutive or random sample of patients (or blood samples) enrolled?	Unclear
	Was a case-control design avoided? Was a range of parasitaemias used?	Unclear
	Did the study avoid inappropriate exclusions?	Unclear
	Risk of bias: Could the selection of patients have introduced bias?	Unclear
	Applicability: Are there concerns that the included patients and setting do not match the review question?	Unclear
DOMAIN 2: Index Test	Were the index test results interpreted without knowledge of the results of the reference standard (if tests were subjective)?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the conduct or interpretation of the index test have introduced bias?	Unclear
	Applicability: Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Unclear
DOMAIN 3: Reference Standard	Is the reference standard likely to correctly classify the target condition?	Yes
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Unclear
	If a threshold was used, was it pre-specified and reasonable (e.g.: qPCR)?	Unclear
	Risk of Bias: Could the reference standard, its conduct, or its interpretation have introduced bias?	Unclear
	Applicability: Are there concerns that the target condition as defined by the reference standard does not match the question?	Unclear
DOMAIN 4: Flow and Timing	Was there an appropriate interval between index test and reference standard (between sample taken)?	Unclear
	Did all patients receive the same reference standard?	No
	Were all patients included in the analysis?	Yes
	Risk of Bias: Could the patient flow have introduced bias?	Low

3x3 tables of test results

Table 67. 3x3 results of Durand et al. (2000)

Reference Test: PCR-RFLP

Index Test: PCR with molecular beacons

Gene: *pfdhfr*

100 samples *P. falciparum*		Reference Test		
		108N	S108	Unknown
Index Test	108N	59	0	0
	S108	0	41	0
	Unknown	0	0	0

Table 68. 3x3 results of Durand et al. (2002)

Reference Test: PCR-RFLP

Index Test: PCR with molecular beacons

Gene: *pfcrt*

130 samples, *P. falciparum*		Reference Test		
		76T	K76	Unknown
Index Test	76T	80	0	0
	K76	0	50	0
	Unknown	0	0	0

Table 69. 3x3 results of Kengne et al. (2003)

Reference Test: PCR-RFLP

Index Test: ASPCR

Gene: *pfdhfr*

56 samples, *P. falciparum*		Reference Test		
		108N	S108	Unknown
Index Test	108N	56	0	0
	S108	0	0	0
	Unknown	0	0	0

Table 70. 3x3 results of Alker et al. (2004)

Reference Test: Sequencing

Index Test: RT-PCR-MGB

Gene: *pfdhfr*

24 samples, 22 malarial, at least 5 *P. falciparum*, 2 non-malarial		Reference Test		
		51I	N51	Unknown
Index Test	51I	5	0	0
	N51	0	0	0
	Unknown	0	0	0

24 samples, 22 malarial, at least 5 *P. falciparum*, 2 non-malarial		Reference Test		
		59R	C59	Unknown
Index Test	59R	4	0	0
	C59	0	1	0
	Unknown	0	0	0

24 samples, 22 malarial, at least 5 *P. falciparum*, 2 non-malarial		Reference Test		
		108N	S108	Unknown
Index Test	108N	5	0	0
	S108	0	0	0
	Unknown	0	0	0

Gene: *pfdhps*

24 samples, 22 malarial, at least 5 *P. falciparum*, 2 non-malarial		Reference Test		
		436A	S436	Unknown
Index Test	436A	0	0	0
	S436	0	5	0
	Unknown	0	0	0

24 samples, 22 malarial, at least 5 *P. falciparum*, 2 non-malarial		Reference Test		
		437G	A437	Unknown
Index Test	437G	3	0	0
	A437	0	2	0
	Unknown	0	0	0

24 samples, 22 malarial, at least 5 *P. falciparum*, 2 non-malarial		Reference Test		
		540E	K540	Unknown
Index Test	540E	4	0	0
	K540	0	1	0
	Unknown	0	0	0

24 samples, 22 malarial, at least 5 *P. falciparum*, 2 non-malarial		Reference Test		
		581G	A581	Unknown
Index Test	581G	1	0	0
	A581	0	4	0
	Unknown	0	0	0

24 samples, 22 malarial, at least 5 *P. falciparum*, 2 non-malarial		Reference Test		
		613S	A613	Unknown
Index Test	613S	0	0	0
	A613	0	5	0
	Unknown	0	0	0

Table 71. 3x3 results of Wilson et al. (2005b)

Reference Test: ASPCR

Index Test: RT-PCR

Gene: *pfcrt*

78 samples, *P. falciparum*[9]		Reference Test		
		76T	K76	Unknown
Index Test	76T	52	0	-
	K76	0	12	-
	Unknown	-	-	-

Table 72. 3x3 results of Farcas et al. (2006)

Reference Test: PCR-RFLP

Index Test: RT-PCR

Gene: *pfcrt*

200 samples, 125 *falciparum*, 40 non-malarial, 22 *vivax*, 10 *ovale*, 3 *malariae*		Reference Test		
		76T	K76	Unknown
Index Test	76T	77	3[10]	-
	K76	0	48	-
	Unknown	-	-	72

[9] - indicates data were not available

[10] Index test results confirmed by sequencing

Table 73. 3x3 results of Juliano et al. (2007)

Reference Test: ASPCR

Index Test: PCR-HSS-HTA

Gene: *pfcrt*

27 samples, *P. falciparum*, Blantyre[7]		Reference Test		
		72-76 CVIET/SVMNK	72-76 CVMNK	Unknown
Index Test	72-76 CVIET/SVMNK	0	1	-
	72-76 CVMNK	0	26	-
	Unknown	-	-	-

60 samples, *P. falciparum*, Mpemba and Madziabango[8]		Reference Test		
		72-76 CVIET/SVMNK	72-76 CVMNK	Unknown
Index Test	72-76 CVIET/SVMNK	0	21	-
	72-76 CVMNK	0	20	19
	Unknown	-	-	-

Table 74. 3x3 results of Juliano et al. (2008)

Reference Test: PCR-MSS-HTA

Index Test: RT-PCR

Gene: *pfdhfr*

85 samples, *P. falciparum*[7]		Reference Test		
		164L	I164	Unknown
Index Test	164L	4	0	-
	I164	0	73	-
	Unknown	-	8	-

52 samples, *P. falciparum*[8]		Reference Test		
		164L	I164	Unknown
Index Test	164L	0	0	-
	I164	0	45	-
	Unknown	-	-	-

Table 75. 3x3 results of Mens et al. (2008)

Reference Test: PCR-RFLP

Index Test: RT-PCR-MCA/FRET

Gene: *pfdhps*

78 samples, *P. falciparum*		Reference Test		
		540E	K540	Unknown
Index Test	540E	36	0	2
	K540	0	27	4
	Unknown	1	4	4

Table 76. 3x3 results of Andriantsoanirina et al. (2009)

Reference Test: Sequencing

Index Test: RT-PCR-HRM

Gene: *pfcrt*

177 samples, *P. falciparum*		Reference Test		
		76T	K76	Unknown
Index Test	76T	20	0	-
	K76	0	39	-
	Unknown	-	-	-

Gene: *pfdhfr*

177 samples, *P. falciparum*		Reference Test		
		51I	N51	Unknown
Index Test	51I	28	3	-
	N51	0	28	-
	Unknown	-	-	-

177 samples, *P. falciparum*		Reference Test		
		59R	C59	Unknown
Index Test	59R	28	3	-
	C59	0	28	-
	Unknown	-	-	-

177 samples, *P. falciparum*		Reference Test		
		108N	S108	Unknown
Index Test	108N	64	1	-
	S108	0	50	-
	Unknown	-	-	-

177 samples, *P. falciparum*		Reference Test		
		164L	I164	Unknown
Index Test	164L	5	0	-
	I164	0	24	-
	Unknown	-	-	-

Gene: *pfdhps*

177 samples, *P. falciparum*		Reference Test		
		436A	S436	Unknown
Index Test	436A	20	3	-
	S436	7	28	-
	Unknown	-	-	-

177 samples, *P. falciparum*		Reference Test		
		437G	A437	Unknown
Index Test	437G	20	3	-
	A437	7	28	-
	Unknown	-	-	-

177 samples, *P. falciparum*		Reference Test		
		540E	K540	Unknown
Index Test	540E	1	0	-
	K540	0	28	-
	Unknown	-	-	-

Gene: *pfmdr1*

177 samples, *P. falciparum*		Reference Test		
		86Y	N86	Unknown
Index Test	86Y	30	3	-
	N86	10	15	-
	Unknown	-	-	-

Table 77. 3x3 results of Juliano et al. (2009)

Reference Test: PCR-MSS-HTA

Index Test: PCR-DIG-HTA

Gene: *pfcrt*

24 samples, *P. falciparum*, Malawi[3]		Reference Test		
		76T	K76	Unknown
Index Test	76T	7	0	0
	K76	1	15	0
	Unknown	0	0	1

31 samples, *P. falciparum*, Madagascar[4]		Reference Test		
		76T	K76	Unknown
Index Test	76T	2	0	0
	K76	0	28	0
	Unknown	0	0	1

Reference Test: PCR-RFLP

31 samples, *P. falciparum*, Madagascar[4]		Reference Test		
		76T	K76	Unknown
Index Test	76T	0	2	0
	K76	0	28	0
	Unknown	0	0	1

Table 78. 3x3 results of Cruz et al. (2010)

From unpublished data

Reference Test: Sequencing

Index Test: RT-PCR-MCA

Gene: *pfdhfr*

46 samples, *P. falciparum*		Reference Test		
		51I	N51	Unknown
Index Test	51I	29	1	0
	N51	0	10	1
	Unknown	4	0	1

46 samples, *P. falciparum*		Reference Test		
		59R	C59	Unknown
Index Test	59R	25	2	0
	C59	4	7	0
	Unknown	5	1	2

46 samples, *P. falciparum*		Reference Test		
		108N	S108	Unknown
Index Test	108N	30	1	0
	S108	1	5	0
	Unknown	6	1	2

46 samples, *P. falciparum*		Reference Test		
		164L	I164	Unknown
Index Test	164L	0	0	0
	I164	0	37	0
	Unknown	0	8	1

Index Test: RT-PCR

46 samples, *P. falciparum*		Reference Test		
		51I	N51	Unknown
Index Test	51I	26	1	0
	N51	0	9	1
	Unknown	7	1	1

46 samples, *P. falciparum*		Reference Test		
		59R	C59	Unknown
Index Test	59R	0	0	0
	C59	29	10	2
	Unknown	5	0	0

46 samples, *P. falciparum*		Reference Test		
		108N	S108	Unknown
Index Test	108N	1	0	0
	S108	3	0	0
	Unknown	33	7	2

46 samples, *P. falciparum*		Reference Test		
		164L	I164	Unknown
Index Test	164L	0	0	0
	I164	0	18	0
	Unknown	0	27	1

Table 79. 3x3 results of Gan and Loh (2010)

Reference Test: RT-PCR

Index Test: PCR-HRM

Gene: *pfcrt*

53 samples, *P. falciparum*		Reference Test		
		72-76 CVIET/SVMNK	72-76 CVMNK	Unknown
Index Test	72-76 CVIET/SVMNK	48	0	0
	72-76 CVMNK	0	5	0
	Unknown	0	0	0

Gene: *pfcytb*

53 samples, *P. falciparum*		Reference Test		
		268N/S/C	Y268	Unknown
Index Test	268N/S/C	0	0	0
	Y268	0	53	0
	Unknown	0	0	0

Table 80. 3x3 results of Muhamad et al. (2011)

Reference Test: RT-PCR (Taqman)

Index Test: RT-PCR (SYBR Green)

Gene: *pfmdr1*

96 samples, *P. falciparum*		Reference Test		
		>1	1	Unknown
Index Test	>1	13	5	7
	1	2	43	26
	Unknown	0	0	0

Table 81. 3x3 results of Moers et al. (2015)

From unpublished data

Reference Test: RT-PCR

Index Test: PCR-PEXT-NALFIA

Gene: *pfcrt*

17 samples, *P. falciparum*, Kenya[5]		Reference Test		
		76T	K76	Unknown
Index Test	76T	9	0	0
	K76	1	5	0
	Unknown	1	1	0

150 samples, 146 *P. falciparum*, 4 non-malarial, MRL[6]		Reference Test		
		76T	K76	Unknown
Index Test	76T	32	0	32
	K76	7	34	2
	Unknown	5	6	32

Reference Test: Sequencing

Gene *pfmdr1*

17 samples, *P. falciparum*, Kenya[5]		Reference Test		
		86Y	N86	Unknown
Index Test	86Y	8	1	1
	N86	0	6	0
	Unknown	0	1	0

150 samples, 146 *P. falciparum*, 4 non-malarial, MRL[6]		Reference Test		
		86Y	N86	Unknown
Index Test	86Y	26	0	2
	N86	2	83	1
	Unknown	7	13	16

17 samples, *P. falciparum*, Kenya[5]		Reference Test		
		184F	Y184	Unknown
Index Test	184F	8	0	1
	Y184	0	7	0
	Unknown	1	0	0

150 samples, 146 *P. falciparum*, 4 non-malarial, MRL[6]		Reference Test		
		184F	Y184	Unknown
Index Test	184F	58	0	0
	Y184	9	42	0
	Unknown	19	4	18

17 samples, *P. falciparum*, Kenya[5]		Reference Test		
		1246Y	D1246	Unknown
Index Test	1246Y	5	0	2
	D1246	1	8	0
	Unknown	0	0	1

150 samples, 146 *P. falciparum*, 4 non-malarial, MRL[6]		Reference Test		
		1246Y	D1246	Unknown
Index Test	1246Y	6	0	0
	D1246	2	89	0
	Unknown	0	18	35

Table 82. 3x3 results of Sharma et al. (2016)

Gene: *pfdhfr*

Reference Test: Sequencing

Index Test: ASPCR

55 samples, *P. falciparum*		Reference Test		
		51I	N51	Unknown
Index Test	51I	6	14	0
	N51	0	35	0
	Unknown	0	0	0

55 samples, *P. falciparum*		Reference Test		
		59R	C59	Unknown
Index Test	59R	38	0	0
	C59	15	2	0
	Unknown	0	0	0

55 samples, *P. falciparum*		Reference Test		
		108N	S108	Unknown
Index Test	108N	39	13	0
	S108	0	3	0
	Unknown	0	0	0

55 samples, *P. falciparum*		Reference Test		
		164L	I164	Unknown
Index Test	164L	4	47	0
	I164	0	4	0
	Unknown	0	0	0

Table 83. 3x3 results of Chahar et al. (2017)

Gene: *pfdhfr*

Reference Test: Sequencing

Index Test: LAMP (gel)

50 samples, *P. falciparum*		Reference Test		
		76T	K76	Unknown
Index Test	76T	36	0	0
	K76	0	14	0
	Unknown	0	0	0

Index Test: LAMP (visible)

50 samples, *P. falciparum*		Reference Test		
		76T	K76	Unknown
Index Test	76T	36	0	0
	K76	0	14	0
	Unknown	0	0	0

Table 84. 3x3 results of Pholwat et al. (2017)

From unpublished data

Reference Test: Sequencing

Index Test: TaqMan array card

Gene: *pfcrt*

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		72-76 CVIET	72-76 CVMNK	Not tested[11]
Index Test	72-76 CVIET	28	0	18
	72-76 CVMNK	0	14	2
	Indeterminate	0	0	23

Gene: *pfcytb*

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		268N/S/C	Y268	Not tested[11]
Index Test	268N/S/C	0	0	0
	Y268	0	42	39
	Indeterminate	0	0	4

Gene: *pfdhfr*

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		51I	N51	Not tested [11]
Index Test	51I	38	0	27
	N51	0	4	3
	Indeterminate	0	0	13

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		59R	C59	Not tested [11]
Index Test	59R	41	0	34
	C59	0	1	0
	Indeterminate	0	0	9

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		108N	S108	Not tested [11]
Index Test	108N	42	0	28
	S108	0	0	0
	Indeterminate	0	0	15

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		164 L	I164	Not tested [11]
Index Test	164L	24	0	10
	I164	0	18	14
	Indeterminate	0	0	19

Gene: *pfdhps*

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		436-437 A-G/S-G	436-437 S-A	Not tested [11]
Index Test	436-437 A-G/S-G	42	0	37
	S-A436-437	0	0	0
	Indeterminate	0	0	6

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		540E	K540	Not tested [11]
Index Test	540E	42	0	19
	K540	0	0	0
	Indeterminate	0	0	24

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		581G	A581	Not tested [11]
Index Test	581G	28	0	12
	A581	0	14	19
	Indeterminate	0	0	12

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		613 S/T	A 613	Not tested [11]
Index Test	613S/T	0	0	0
	A613	0	42	35
	Indeterminate	0	0	8

Gene: *pfk13*

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		458 Y	N 458	Not tested[11]
Index Test	458Y	0	0	0
	N458	0	42	35
	Indeterminate	0	0	8

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		493 H	Y 493	Not tested[11]
Index Test	493H	0	0	0
	Y493	0	42	36
	Indeterminate	0	0	7

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		539 T	R 539	Not tested[11]
Index Test	539T	0	0	0
	R539	0	42	35
	Indeterminate	0	0	8

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		543 T	I 543	Not tested[11]
Index Test	543T	0	0	0
	I543	0	42	32
	Indeterminate	0	0	11

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		561 H	R 561	Not tested[11]
Index Test	561H	1	0	0
	R561	0	41	37
	Indeterminate	0	0	6

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		580 Y	C 580	Not tested[11]
Index Test	580Y	0	0	0
	C580	0	42	36
	Indeterminate	0	0	7

Gene: *pfmdr1*

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		86Y	N86	Not tested [11]
Index Test	86Y	5	0	0
	N86	0	37	36
	Indeterminate	0	0	7

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		184F	Y184	Not tested [11]
Index Test	184F	19	0	17
	Y184	0	23	15
	Indeterminate	0	0	11

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		1034C	S1034	Not tested [11]
Index Test	1034C	0	0	0
	S1034	0	42	32
	Indeterminate	0	0	11

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		1042D	N1042	Not tested [11]
Index Test	1042D	1	0	0
	N1042	0	41	32
	Indeterminate	0	0	11

87 samples, 85 *P. falciparum*, 1 *P. vivax*, 1 *P. ovale*		Reference Test		
		1246Y	D1246	Not tested [11]
Index Test	1246Y	4	0	0
	D1246	0	38	33
	Indeterminate	0	0	10

[11] Not tested by reference test

Excluded studies

Table 85. Excluded studies

Study	Title
Nair et al. (2002)	Rapid genotyping of loci involved in antifolate drug resistance in *Plasmodium falciparum* by primer extension
Gil et al. (2003)	Detection of atovaquone and Malarone resistance conferring mutations in *Plasmodium falciparum* cytochrome b gene (*cytb*)
Purfield et al. (2003)	Real time PCR for analysis of *pfmdr1* mutations
Wilson et al. (2005b)	Prevalence of *pfcrt* mutations in Congolese and Malawian *Plasmodium falciparum* isolates as determined by a new Taqman assay
Mishra et al. (2006)	An efficient PCR--SSCP-based method for detection of a chloroquine resistance marker in the *pfcrt* gene of *Plasmodium falciparum*
Carnevale et al. (2007)	A multiplex ligase detection reaction-fluorescent microsphere assay for simultaneous detection of single nucleotide polymorphisms associated with *Plasmodium falciparum* drug resistance
Crameri et al. (2007)	Rapid microarray-based method for monitoring of all currently known single-nucleotide polymorphisms associated with parasite resistance to antimalarial drugs
Zhang et al. (2008)	Multiplex PCR and oligonucleotide microarray for detection of single-nucleotide polymorphisms associated with *Plasmodium falciparum* drug resistance
Khairnar and Pillai (2009)	A rapid pyrosequencing method for detection of single nucleotide polymorphism (SNPs) associated with antimalarial resistance
Steenkeste et al. (2009)	FlexiChip package: an universal microarray with a dedicated analysis software for high-throughput SNPs detection linked to anti-malarial drug resistance
Matrevi et al. (2010)	Chloroquine resistance haplotypes in the *pfcrt* and *mdr1* genes of Ghanaian isolates using PCR and sequence specific oligonucleotide isolates using PCR and sequence specific oligonucleotide probe-enzyme linked immunosorbent assay (SSOP-ELISA)
Mugasa and Ranford-Cartwright (2010)	New Lab-on-a-Chip for fast diagnosis of human malaria species and detection of drug resistance
Akter (2012)	Genotyping of *Plasmodium falciparum* in Bangladesh using Antigenic Polymorphic markers and comparison with anti-malarial drug resistance markers genotype
Daniels et al. (2012)	Rapid, Field-Deployable Method for Genotyping and Discovery of Single-Nucleotide Polymorphisms Associated with Drug Resistance in *Plasmodium falciparum*
Kamau et al. (2012)	Development of a TaqMan Allelic Discrimination Assay for detection of Single Nucleotides Polymorphisms associated with anti-malarial drug resistance
LeClair et al. (2013)	Optimization of a ligase detection reaction fluorescent microsphere assay for the characterization of resistance-mediating polymorphisms in African samples of *Plasmodium falciparum*
Morris et al. (2013)	Rapid diagnostic tests for molecular surveillance of *Plasmodium falciparum* malaria - assessment of DNA extraction methods and field applicability
Nankoberanyi et al. (2014)	Validation of the ligase detection reaction fluorescent microsphere assay for the detection of *Plasmodium falciparum* resistance mediating polymorphisms in Uganda
Thompson et al. (2015)	High-resolution melt analysis to compare the efficacy of an investigational antimalarial (AQ-13) with coartem (artemether-lumefantrine) for treatment of uncomplicated malaria due to chloroquine-resistant vs. chloroquine-susceptible *Plasmodium falciparum*
Ndiaye et al. (2017)	High resolution melting: a useful field-deployable method to measure *dhfr* and *dhps* drug resistance in both highly and lowly endemic *Plasmodium* populations

SWAR: Finding eligible studies efficiently

Objective of this SWAR: To examine the sources of eligible studies, and the most efficient use of those sources.

Study area: Study identification

Sample type: Reviewers

Estimated funding level needed: Low

Background: Higgins and Green (2008) and CRD (2009) provide a wealth of assistance in choosing search sources. However, there is little evidence available about the efficiency of searching different sources (Booth, 2010, Ross-White and Godfrey, 2017). The efficient use of sources is particularly important for reviews with limited time resources, search strategies low in specificity or asking questions for which the evidence may develop rapidly.

Interventions and comparators: As this study is an observational study, neither an intervention nor a comparator will be used.

Index Type: Full review

Primary outcome measures: The sources searched and, for each, the total number of search results, and the number of eligible studies identified. The results can be recorded in the template provided:

Figure 13. Template for recording the source of eligible studies

	Study 1	Study 2	Study 3	Number of eligible studies	Number of unique eligible studies	Number of search results
Source A	✓		✓	2	1	100
Source B	✓	✓	✓	16	0	7086
Source C	✓	✓	✓	16	0	4122

Analysis plans: The data collected for each review can be published alongside the example search strategy required by PRISMA reporting guidelines. The results could also be collated in a publicly accessible database with the title, abstract and PROSPERO number of the review. This will allow future reviewers to compare published reviews and sources of eligible studies with their planned review and take a more informed decision when choosing which sources to use.

Possible problems in implementing this SWAR:

The simplest way to carry out a systematic review is to use reference management software to delete duplicate search results. However, in order to gather data for this review, all results are required. The easiest way to conduct this SWAR may be to repeat the search, or to start by creating two identical reference libraries, only one of which has duplicate results removed.

The data generated for each review may be specific to certain subjects, or to certain combinations of subjects, and not be highly applicable to reviews in other areas.

The more reviewers collect and publish this data as a part of their reviews, the more useful the collected data will be; this SWAR has limited use if it is used in few reviews, or in few types of review question.

Diagnostic Accuracy Study Protocol

Diagnostic accuracy study to assess the sensitivity and specificity of Nanomal to detect *Plasmodium falciparum* drug resistance markers in patients with malaria: a protocol

Abstract

Background *Plasmodium* is a major cause of morbidity and mortality globally, with an estimated Infection with *Plasmodium* caused 148-304 million clinical cases of malaria, and 235-639 thousand deaths in 2015. Fast and accurate diagnosis can reduce morbidity and mortality but access to diagnosis differs greatly. With emerging and spreading drug-resistance, diagnosis and tracking of resistance is increasingly important in enabling effective treatment. Nanomal is a diagnostic test for malaria, designed to identify *Plasmodium* species and key drug-resistance markers.

Objectives In this study we will test Nanomal for the first time in a clinical setting. We will determine the sensitivity and specificity of Nanomal for detection of markers of antimalarial resistance compared to a reference standard: Real-Time Polymerase Chain Reaction (RT-PCR). We will determine the Positive Predictive Value (PPV) and Negative Predictive Value (NPV) in this setting.

Methods This blinded diagnostic study will take place in the Albert Schweitzer Hospital in Lambaréné, Gabon, comparing the results of RT-PCR and Nanomal tests in 168 patients presenting with malaria in 2018.

Findings The results will guide the decision whether to deploy Nanomal as a diagnostic tool to replace Rapid Diagnostic Tests (RDTs) and to contribute to surveillance of antimalarial resistance, or to carry out further optimisation before re-testing Nanomal in a diagnostic accuracy study.

This study will be registered with ISRCTN Registry (ISRCTN, 2015). The full protocol and the completed study will be published in an open-access journal and reported following 2015 STARD guidance (Bossuyt et al., 2015). All sources of funding and possible conflicts of interest will be declared.

Lay Summary

Why do we need this study?

Every year, millions of people suffer from malaria, and hundreds of thousands of people are killed by it. Malaria is treatable, but many people live a long way from medical centres where malaria can be diagnosed accurately. Another problem is drug resistance. Some parasites have developed a resistance to the drugs used to treat malaria, and so it is important to work out what drugs will work for each patient, and to monitor drug resistance to try to stop it from spreading. The best tests for diagnosing malaria are Polymerase Chain Reaction (PCR) and Real-Time Polymerase Chain Reaction (RT-PCR), which look in the patient's blood for the DNA of the parasite that causes malaria. These are accurate tests, but are expensive, require highly trained staff, and cannot easily be transported to the places where people who might have malaria live. There are Rapid Diagnostic Tests (RDTs) that are easier to use and transport, and are cheaper, but these are less accurate.

What are we testing, and in whom?

This study will look at a new test, Nanomal, for diagnosing malaria and detecting drug resistance. Nanomal is cheap, simple to use, requires just one drop of blood, and can easily be transported anywhere in the world. At St. George's, University of London, we have examined Nanomal using samples in the laboratory, but have not yet examined it using samples from patients with malaria. We need to find out how good this test is at detecting drug resistant parasites that cause malaria in real patients. This research will compare the best test we have now, RT-PCR, with the new test, Nanomal. The research will take place at The Albert Schweitzer Hospital in Lambaréné, Gabon, in 2016. 168 patients with malaria will be tested.

What will we do with the results?

When all the results are available we will have enough information to make a decision about what to do next with Nanomal. If Nanomal is a useful test, then we can start using it to diagnose people, to treat patients earlier and more effectively.

Introduction

Plasmodium is a major cause of morbidity and mortality globally, with 148-304 million clinical cases of malaria, and 235-639 thousand deaths in 2015 (WHO, 2015d). Fast and accurate diagnosis can reduce morbidity and mortality (McGready et al., 2012). Access to diagnosis differs greatly; worldwide, 78% of suspected malaria cases presenting in the public sector receive a diagnostic test for malaria. In sub-Saharan Africa where the majority of cases and deaths occur, between 6-53% of febrile children seeking care receive a diagnostic test for malaria. Many people are unable to access healthcare facilities at all (WHO, 2015d). However, some *Plasmodium* parasites are resistant to other antimalarial chemotherapies and responsible for increased mortality (Trape et al., 1998, Korsinczky et al., 2000, Nosten et al., 2000, Bousema et al., 2003, Mullick et al., 2011).

Artemisinin Combination Therapies and artemisinins are the World Health Organization (WHO) recommended first-line treatments for most patients with uncomplicated *falciparum* malaria, or with severe malaria, including those in Gabon (WHO, 2015b). However, resistance to artemisinins has recently emerged in the Greater Mekong sub-region; failure of ACTs may also be due in part to factors, such as resistance to partner drugs (Venkatesan et al., 2014, Lu et al., 2017, Mukherjee et al., 2017).

Numerous malaria diagnostics have already been developed and tested in clinical settings, however, needs differ between settings depending on factors such as transmission and available resources. Diagnostic tools do not currently meet the needs of low resource settings, and so uptake of diagnostics is low (Krampa et al., 2017, Roth et al., 2016). The development of adequate diagnostic tools is a key component of the WHO Global Technical Strategy for Malaria 2016-2030 (WHO, 2015a).

Existing diagnostics for malaria fall into 3 main categories: microscopy, RDTs and nucleic acid amplification.

Microscopy

Microscopy is the mainstay of diagnosis, widely used in endemic and non-endemic countries. When carried out by expert microscopists it can produce a sensitive, specific, and speciated diagnosis within an hour. However, quality of expert microscopy is variable and can miss mixed and low parasitaemia infections (Gupta et al., 2010, McKenzie et al., 2003, Mukadi et al., 2011). Microscopy cannot detect antimalarial resistance.

RDTs

RDTs (lateral flow devices using immunochromatography to detect soluble malarial antigens) are designed for use in low resource settings. RDTs have been reviewed compared to other diagnostics and for non-*falciparum* infections (Abba et al., 2011, Abba et al., 2014). These reviews found that sensitivity and specificity varies little between RDTs. The main differences are in their capabilities; some are able to detect only one species, some are able to detect multiple species but not distinguish between them, and some are not able to distinguish between recent and current infections. RDTs are not sensitive enough to detect *Plasmodium* infections at low parasitaemias (McMorrow et al., 2011). Current RDTs cannot detect antimalarial resistance.

Nucleic acid detection

Different PCR methods including RT-PCR are now routine in many laboratories. A variety of PCR techniques are used to detect *Plasmodium* with very high but differing levels of sensitivity and specificity (Alemayehu et al., 2013, Burrow, 2017). RT-PCR enables diagnosis within a few hours, including detection of drug resistance markers.

Requirements of a diagnostic tool for malaria

None of the existing diagnostics for antimalarial drug resistance meet the essential requirements, which are:

- **To be a sensitive and specific diagnostic test**. A huge number of tests are carried out to diagnose presence or absence of *Plasmodium*, even small deficiencies in sensitivity and specificity lead to hundreds of thousands of false negative and false positive results. If tests to diagnose and monitor antimalarial resistance are incorporated into *Plasmodium* diagnostic tests then the same applies to detection of antimalarial resistance.
- **To enable prescription of an effective treatment.** Due to widespread drug resistance, genotyping of drug resistance markers is required to identify effective treatment options for individual patients. Using an effective first-line treatment increases the chance of preventing death or long-term sequelae for an individual patient, and reduces infectious transmission (White, 2008).
- **To reduce the development and spread of drug resistant malaria.** Reducing and changing selective pressures on circulating infections allows mutant genotypes maintained by drug pressures to decline, and wildtype (or other resistant) genotypes to increase in prevalence. This may allow treatments that are currently not useful to become efficacious again (Ord et al., 2007).
- **To collect and maximise the utility of the data generated by diagnosis.** Malaria cases, species and drug resistance need to be tracked to understand the epidemiology of malaria in order to eradicate malaria (Moss et al., 2015).

Diagnostics for use outside of well-resourced hospitals also need to be:

- Affordable for wide-spread use in developing countries
- Comprised of components which are stable in a variety of environments
- Easily transported to rural and difficult to access areas
- Functional without a constant power source
- Simple to use to enable correct operation

- Simple to understand the results to enable correct interpretation and treatment
- Fast to produce results; sometimes patients will not, or cannot, wait hours or days for a result

Nanomal

Nanomal (QuantumDx, UK) is designed to meet these requirements and replace RDTs. It is able to diagnose malaria and determine species and drug resistance markers within 20 minutes. It uploads all data to an online database. It will cost less than $5 per test. The components and reagents are stable under a wide range of abiotic stresses. The device is small and light enough to hold in one hand, and easily transported with a mobile charging station. To operate it requires the addition of a finger-prick of blood to one end of the device. The readout is a qualitative assessment of presence of *Plasmodium*, species, and (in addition to individual drug resistance makers) an assessment of which drugs the infection is resistant to. Unpublished *in vitro* data from St. George's, University of London shows that the sensitivity of Nanomal for detecting the presence of *Plasmodium*, defined as a positive result for any of the four species, is 90-100% and specificity is 95-100%. The additional capabilities of Nanomal compared to other diagnostic tools, and the promising *in vitro* data justify research into Nanomal in a clinical setting.

Outcomes

The primary outcomes of this study are the sensitivity and specificity of Nanomal to detect resistant alleles at position 76 in the *Plasmodium falciparum chloroquine resistance transporter* (*pfcrt*) gene.

Methods

Study Design

This is a prospective diagnostic accuracy study. 168 participants will be recruited at the Albert Schweitzer Hospital in Lambaréné, Gabon. Potentially eligible participants will be identified by their status as patients who have been referred for malaria testing. They will be confirmed as eligible when expert microscopists confirm the presence of *Plasmodium falciparum*. This study will start in 2018 and will continue until 168 eligible participants have been enrolled, which is expected to take <9 months (The Albert Schweitzer Fellowship, 2013, WHO, 2015d). Consecutive sampling will be used and both index (Nanomal) and reference (RT-PCR) tests used for all participants. We chose one marker in one gene responsible for the majority of treatment failures due to chloroquine drug resistance, also conferring resistance to the artemisinin partner drugs in Artemisinin Combination Therapies (ACTs), the current first line treatments (Staines and Krishna, 2012). Diagnosis of resistance will be the machine readout reporting a threonine (T) at position 76, rather than Lysine (K).

Inclusion and Exclusion Criteria

To reduce the risk of bias the inclusion criteria are as wide as possible: all patients presenting to a clinician, whom is suspected by the clinician to have malaria, for any reason, and has been referred for malaria testing which has come back with a microscopy result positive for *Plasmodium falciparum*. All patients with known or suspected concurrent infections and conditions will be eligible.

Recruitment and Consent

With patient and public involvement (PPI) we will create clear and comprehensive information leaflets about the study. These will be available in English, French, and Bantu languages as chosen by PPI reviewers. A health worker will be available to explain the research and support patients and their families in participation in the trial. If recruitment is slower than expected, advice will be sought on what the problems may be, and possible solutions. Many patients may be children, or adults too ill to

consent. In these cases consent will be sought immediately from a legal representative, and failing that, later from the patient themselves. As the study is minimally invasive, this is justified.

Clinical management of participants

The results of the Nanomal test will not be used to guide clinical management of the participants. As the sensitivity and specificity of Nanomal is not established in a clinical setting, there is an increased risk of not treating patients with malaria, or wrongly treating patients without malaria, based on misleading Nanomal results. The standards of diagnosis and treatment are not compromised; participants will receive the same diagnosis and treatment as the patients not enrolled in this trial. Should RT-PCR results become available while the patient is still unwell, they should be provided to the clinician and used to inform care.

Index Test

Nanomal is the index test. More information can be found in “Laboratory Methods”.

Reference Test

RT-PCR is the reference test; RT-PCR is one of the most specific and sensitive methods of diagnosis (Chiodini, 2014, Burrow, 2017). We chose not to use sequencing as the reference test as this has a similar accuracy to RT-PCR and is more expensive and slow to produce results.

Blinding

Nanomal, and RT-PCR will be assessed and recorded by different members of staff, each blinded to the results of the other test. However, the member of staff assessing and recording the results of the Nanomal test will have access to the clinical information about the patient that was available when they were enrolled into the trial. The member of staff carrying out, analysing and recording the results of the RT-PCR tests will have access to the results of any other RT-PCR tests that are being conducted. The Nanomal results are a qualitative objective readout, and RT-PCR test results are objective with pre-defined thresholds and analysis methods, so the risk of observer bias is low.

Outcomes

As this is the first test of Nanomal in a clinical setting we felt that the primary outcome should be determining sensitivity and specificity of one resistance marker of clinical importance. A larger study would be required for sufficient power to determine specificity and sensitivity for each of the species and drug resistance markers.

Ethics

Permission for the study will be sought from Comité d'Éthique Régional Indépendant de Lambaréné (CERIL), and from the St. George's University of London Research Ethics Committee in the UK. A group will be established to monitor the trial, give oversight, and review any adverse events in line with the UK Clinical Trials Regulations 2004 and The Declaration of Helsinki.

Laboratory Methods

Samples for Nanomal (finger-prick) and RT-PCR (5ml vacutainer) will be taken within 1 hour to prevent bias caused by temporal changes in circulating parasitaemia.

Index Test: Nanomal

The Nanomal sample will be taken by a trained healthcare worker, who will also operate Nanomal, read out the results and record them on a paper CRF. The method by which Nanomal works is currently confidential intellectual property of the institutions and companies collaborating to create it, and so it is not possible to describe this test in enough detail to allow understanding or replication. The information available can be viewed on the Nanomal website (The Nanomal Consortium). The Nanomal test will only be carried out once for each participant.

Reference Test: RT-PCR

The blood sample routinely taken for microscopic analysis will also be used for immediate storage at -20°C, followed by DNA extraction with a commercial extraction kit (QIAamp DNA Blood Mini Kit,

Qiagen) following manufacturer instructions and DNA stored at −80°C for further analysis by RT-PCR. All laboratory work will be carried out by trained molecular biologists in a GLP certified lab. RT-PCR will only be carried out once for the primary analysis. RT-PCR may be repeated for indeterminate samples to confirm whether discrepancies between tests are due to Nanomal performing better than RT-PCR.

For *pfcrt* amino acid 76 will be determined by RT-PCR (Gadalla et al., 2010, Sutherland et al., 2007).

Analysis of Tests

Test Positivity and Negativity - Nanomal

Test results of Nanomal are determined internally by the device. It provides an on-screen readout which states:

- "*Plasmodium*" OR "*vivax*" OR "*malariae*" OR "*ovale*" if a *Plasmodium* species is detected
- "Mutation X in gene Y" if a mutation is detected
- "Wildtype X in gene Y" if a wildtype is detected
- "Negative" if no *Plasmodium* species is detected
- "Uninterpretable" if species detection can be classed neither as positive or negative
- "Uninterpretable X in gene Y" if neither mutation or wildtype can be detected

Test Positivity and Negativity – RT-PCR

Test results of RT-PCR are determined using a pre-defined cut-off for maximum Cycle threshold (Ct) and minimum fluorescence to eliminate false positives caused by contamination. Negative and positive controls will be used and results classed as uninterpretable if negative control results are positive, or positive control results are negative. If a resistance marker probe has over the minimum fluorescence and under the maximum Ct, the species test is positive for that allele. If the probe has

under the minimum fluorescence, or over the maximum Ct, the species test is negative. In any other circumstance the result is uninterpretable.

Other results – Nanomal, RT-PCR

Using both methods, mixed drug resistance genotypes are detectable, and will be counted as resistant.

There will be no exploratory categories of test result.

Management and Analysis of Results

Data Management

Data will be collected directly onto paper Case Report Forms (CRFs), which will be double-entered onto an electronic database. Database consistency checks and queries will be used, and 10% of the source files reviewed. The variables collected will include: date of birth, initials, sex, concurrent infections, conditions and medications, RT-PCR results for the drug resistance marker *pfcrt* K76T, and Nanomal results for drug resistance SNP (wildtype or mutant). Data will be cleaned and coded after prior to analysis. Data and samples will be assigned pseudonyms, data archived for future analysis, and managed in line with the UK Data Protection Act 1998.

Missing and Uninterpretable Results

Missing Nanomal results will be considered for their cause, analysed together with uninterpretable results, and presented broken down by RT-PCR result. Although it has been recommended that only valid inconclusive results be considered as part of the analysis, to exclude any missing or uninterpretable results from the analysis could lead to over-estimation of sensitivity and specificity (Shinkins et al., 2013). Missing RT-PCR results (of which we expect very few) will be presented, but removed from the primary analyses, along with the corresponding Nanomal results. There is no clear mechanism by which the number of uninterpretable and missing results will be affected by disease status.

Presentation of Results

The flow of participants through the study will be shown using a STARD diagram (Bossuyt et al., 2015). Demographic and clinical characteristics of participants, including severity of disease and other diagnoses will be shown in a table. The primary outcomes results will be presented in a table.

Statistical Analysis

Stata 14 will be used to calculate sensitivity and specificity and exact binomial 95% confidence intervals (CI) (Clopper and Pearson, 1934). Positive Predictive Values (PPV), Negative Predictive Values (NPV) and likelihood ratios will also be calculated.

Sample Size

From unpublished *in vitro* data, the best estimate of sensitivity of Nanomal is 90-100%, and specificity is 95-100%. Data on test positivity was not available from the site. Approximately 30% of microscopy tests in the public sector in sub-Saharan Africa returned a positive result for *Plasmodium falciparum* (WHO, 2015d). Surveys of resistance in Lambaréné have found a 97% prevalence of *pfcrt* 76T resistant allele (Frank et al., 2011). Using these data, with 95% confidence intervals and powered for 80% accuracy, the following calculations in Figure 14 show how samples sizes were determined:

Figure 14: Sample size calculations

Sensitivity

$$\text{true positives + false negatives} = \frac{1.96^2 \times (\text{sensitivity} \times (1 - \text{sensitivity}))}{\text{accuracy}^2}$$

$$\text{true positives + false negatives} = 8.6436$$

$$\text{sample size} = \frac{\text{true positives + false negatives}}{\text{prevalence}}$$

$$\text{sample size} = 8.9$$

Specificity

$$\text{false positives + true negatives} = \frac{1.96^2 \times (\text{specificity} \times (1 - \text{specificity}))}{\text{accuracy}^2}$$

$$\text{false positives + true negatives} = 4.6$$

$$\text{sample size} = \frac{\text{false positives + true negatives}}{\text{prevalence}}$$

$$\text{sample size} = 152.1$$

A power of 80% is sufficiently likely to determine an accurate result. Increasing the power further made the study unfeasibly large. In order to allow for missing and uninterpretable results, and drop-outs from the study, the sample size was increased by 10%. It is unlikely that patients will drop out as the intervention is minimally invasive. However, given the lack of experience of use of the Nanomal device in a clinical setting, it seems wise to allow a moderate margin for missing results.168 patients will be enrolled.

Scope of this Study

This study is designed based on the information available from the manufacturer of Nanomal. This information is very limited in quantity, likely to be incomplete and perhaps biased towards favouring the device. However, we have sufficiently powered the study to cope with lower estimates of sensitivity and specificity.

This study will be limited by its sample size; in Gabon, some species and drug resistance markers will not appear at a high enough frequency to determine the sensitivity and specificity of Nanomal for them. However, the study is adequately large for the first question of sensitivity and specificity for detection of *pfcrt* 76.

Diagnostic variability will not be studied. The resources required to carry out a study of diagnostic variability include multiple finger-pricks from each participant and an investment in multiple prototype Nanomal devices. To carry out a study of subgroup variability would require a much larger study. Neither is justified at such an early stage of clinical testing.

The data will be applicable to a hospital setting in an area of high transmission, while the target setting is rural, in areas of low and high transmission. Prevalence of malaria and parasitaemia are likely to be higher in this study than in the intended setting. Patients in hospital are likely to over-represent the number of severe cases, cases in children, and cases of multiple infection. They are likely to underrepresent fatal cases or short, mild, or asymptomatic cases. This may mean that the sensitivity of Nanomal will be lower in a rural setting than in a hospital setting.

The site of the study has been chosen for its high prevalence of endemic malaria, established staff and equipment required for such a study, and a vast amount of experience in carrying out clinical trials. The results of this study will not be precisely those that would have been found in a different setting, but they will provide an accurate assessment of sensitivity and specificity of Nanomal for detection of *pfcrt* 76 in this setting.