Summary of discordant results between rapid diagnosis tests, microscopy, and polymerase chain reaction for detecting *Plasmodium* mixed infection: a systematic review and meta-analysis

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Malaria rapid diagnostic tests (RDTs) are widely used to detect malaria parasites among patients who suspected malaria infections in malaria-endemic areas where microscopy is unavailable. Nevertheless, little is known about the performance of RDTs in detecting *Plasmodium* mixed infections. The present study aimed to evaluate the discordant results between RDTs and microscopy/polymerase chain reaction (PCR) in detecting *Plasmodium* mixed infections. The PubMed (MEDLINE), Web of Science, and Scopus databases were systematically reviewed to identify related studies that reported the performance of RDTs in detecting *Plasmodium* mixed infections. Studies were grouped according to the different RDT types including RDT type 2 (pf-HRP2/pan-aldolase), RDT type 3 (pf-HRP2/pan-pLDH), RDT type 4 (Pf-LDH/pan-pLDH), RDT type 5 (Pf/Pv-pLDH), and RDT type 6 (pf-HRP2/Pv-pLDH) for subgroup analysis. The estimates of the different proportions in each analysis group that were visually summarized in a forest plot showed the odds ratio (OR) and 95% confidence interval (CI). Plots were drawn using RevMan (version 5.3; Cochrane Community). Twenty-eight studies were included in the present study. Overall, the meta-analysis showed that RDTs could detect a significantly higher proportion of *Plasmodium* mixed infections than microscopy (p = 0.0007, OR = 3.33, 95% CI 1.66–6.68). Subgroup analysis demonstrated that only RDTs targeting Pf-specific histidine-rich protein 2 (HRP2)/pan-specific lactate dehydrogenase (LDH) could detect a significantly higher proportion of *Plasmodium* mixed infections than microscopy (p = 0.004, OR = 8.46, 95% CI 2.75–26.1). The subgroup analysis between RDTs and PCR methods demonstrated that RDTs targeting Pf-specific HRP2/Pv-specific LDH could detect a significantly lower proportion of *Plasmodium* mixed infections than PCR methods (p = 0.0005, OR = 0.42, 95% CI 0.26–0.68). This is the first study to summarize the discordant results between RDTs and microscopy/PCR in detecting *Plasmodium* mixed infections. Malaria RDTs targeting Pf-HRP2/pan-pLDH could detect a higher proportion of *Plasmodium* mixed infections than microscopy, while RDTs targeting Pf-HRP2/Pv-specific LDH could detect a lower proportion of *Plasmodium* mixed infections than PCR methods. The results of this study will support the selection and careful interpretations of RDTs for a better diagnosis of *Plasmodium* mixed-species infections and appropriate treatment of malaria patients in endemic and non-endemic settings.

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Malaria is a public health problem reported worldwide especially in the African region (213 million or 93%), with an estimated 405,000 deaths from malaria globally in the year 2018. Human malaria is caused by five species of *Plasmodium* spp. including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. Microscopy and rapid diagnostic tests (RDTs) are diagnostic tools to confirm the diagnosis in patients suspected of having malaria. Currently, the microscopic method is the gold standard for malaria detection and diagnosis. However, it is imperfect by nature, especially in the identification of mixed-infections among residents in community-endemic areas. Sub-microscopic mixed-infections with low parasite density are commonly missed by microscopic methodologies. Therefore, mixed infections of *Plasmodium* spp. are often unrecognized and underestimated due to the low detection rate by microscopy (2%)4,5. Misdiagnosis of *Plasmodium* mixed infections can lead to anti-malarial drug resistance and the development of severe malaria. RDTs are easy to use and cost effective. They play a crucial role in the control of malaria when microscopy is unavailable and are convenient to use in field surveys or remote areas where laboratory capacity is limited. RDTs are immunochromatographic lateral flow devices of which commonly targeting histidine-rich protein-2 (HRP2), lactate dehydrogenase (LDH), and aldolase RDTs for rapid malaria detection4,5. RDTs targeting HRP2 are specific for the detection of *P. falciparum*, while RDTs targeting LDH can be used for the detection of *P. falciparum*, *P. vivax*, or pan-specific (e.g., four *Plasmodium* species) LDH antibodies; aldolase is another common target for RDTs to detect all *Plasmodium* species2,7,10. Recently used commercial dipsticks for the detection of HRP-2 include PfHRP2 CareStart11-13, SD Bioline Malaria Ag Pf14,15, and one recently used for the detection of pLDH is CareStart pLDH(pan)15. A recently used commercial dipstick for the detection of Pan-aldolase is ParaHit Total, while a recently used commercial dipsticks for the detection of *P. vivax* aldolase is mAb IC3-12 F1017. Recently used commercial dipsticks for the detection of HRP-2/pLDH include SD BIOLINE Malaria Ag Pf/Pan16 and CareStart malaria HRP2/pLDH (Pf/Pan) Combo test18. Finally, recently used commercial dipsticks for the detection of HRP-2/pan-aldolase include Malaria Pf/Pan Rapid Test Device Acon19 and ParaHIT Total Dipstick20.

Even though a large number of RDTs are available for malaria detection, the widespread use of RDTs causes the missed detection of mixed-species infections in individuals21. Moreover, their performance for the detection of mixed-species infections is less requires well more comprehensive studies. Since the accurate detection of mixed-species infections of malaria is very critical for successful malaria control programmes, the objective of this systematic review and meta-analysis was to summarise and analyse the performance of malaria RDTs in detecting *Plasmodium* mixed infections. This study aims to highlight the big knowledge gap on the performance of malaria RDTs in detecting these mixed-species infections and to help make informed decisions on the use of RDTs for prompt treatment, which will help eliminate malaria in endemic and non-endemic areas.

**Methods**

**Search strategy.** Searches of Medline (PubMed), Web of Science, and Scopus were systematically performed using the search terms provided in Supplementary Table S1. The searches were limited to the English language. Searches were carried out and finished on 1 April 2020. All reference lists of all eligible and included studies as well as Google Scholar search was performed to further increase the number of included articles for review.

**Definition of malaria RDTs and microscopy.** Types of malaria RDTs were classified according to the study by Bell et al.7. They classified malaria RDTs into seven types according to the antigen used in the reagent strip, including type 1 (HRP2 (falciparum-specific), 2 (pf-HRP2/pan-aldolase), 3 (pf-HRP2/pan-pLDH), 4 (Pf-LDH/pan-pLDH), 5 (Pf/Pv–pLDH), 6 (pf-HRP2/Pv-pLDH), and 7 (aldolase). RDTs types 2, 3, 4, 5 and 6 can detect mixed or concurrent infections. Interpretation of *Plasmodium* mixed-infections by RDT was based on details provided by authors of the included studies. The gold standard for malaria detection is still microscopy where the examinations of thin and thick blood films lead to the demonstration of malaria parasites.

**Inclusion and exclusion criteria.** Cross-sectional studies that reported the number of *Plasmodium* mixed infections evaluated by any of the five types of RDTs (types 2, 3, 4, 5 and 6) in comparison to microscopy or PCR were included in the present study. Studies reporting the results of RDTs and microscopy from the same patient samples or those reporting the results of RDTs and PCR from the same patient samples were included in the study. The following types of literature were excluded; studies that reported mixed-infections only for RDTs but did not report microscopy or PCR, incomplete data, no RDT results, co-infections with other agents, experimental studies, review articles, case reports and case series, polymorphism/mutation studies, knowledge about malaria/practice assessments, animal/mosquito studies, studies of haematological alterations, guidelines, and clinical drug trials. Studies with no full text and present data in the local language were also excluded.

**Data extraction.** All studies acquired through the search were stored in EndNote reference manager software (version X9; Clarivate Analytics). The data extractions started with screening the titles and abstracts after
duplicate studies removed. Studies that were not related to the inclusion criteria were excluded. Then, the studies were screened for full-text articles, and those that did not comply with eligibility criteria were excluded with tags indicating the reason for exclusion. The data from full-text articles that passed the inclusion and exclusion criteria were then exclusively examined and extracted by two independent authors (MK and KUK) using an Excel spreadsheet for further analysis. Any inconsistencies relating to included studies and data extraction were resolved by a third or a fourth reviewer (FRM or GDM).

**Statistical analysis.** Studies were grouped (subgroup) according to the different RDT types for comparative analysis. The meta-analysis of the proportion of the number of *Plasmodium* mixed infections per the total number of total malaria positives were performed as follows: (1) the summary estimate of the difference in the proportion (odds ratios, ORs) of RDTs to detect mixed infections compared with microscopy and (2) the summary estimate of the difference in the proportion (ORs) of RDTs to detect mixed infections compared with PCR methods were estimated. The subgroup analysis of RDT types, blood collection methods (finger prick or venipuncture), and types of *Plasmodium* mixed species confirmed by PCR were analysed in the present study. All analyses were conducted using Review Manager Version 5.3 (Cochrane, UK). The statistical analysis used to calculate the difference between groups was the Mantel–Haenszel test with a random-effects model. The meta-analysis for each study and the overall studies are presented with OR and 95% confidence intervals (CIs) as effect measures and summarized in forest plots. Cochrane’s Q test and Higgins’s I² statistics were performed to assess the heterogeneity of the included studies.

**Quality of included studies.** The quality of the individual studies included in the present study was assessed by the Quality Assessment of Diagnostic Accuracy Studies (QUADAS)25. The tool includes 4 domains including the following: (1) report the review question, (2) develop review-specific guidance, (3) review the published flow diagram, and (4) judge bias and applicability. Each domain was assessed in terms of the patient selection, index test, reference standard, and flow timing. Patient selection was the method of patient selection reported in the included studies. The index test was the RDT method that was conducted and interpreted in the included studies. The reference standards were microscopy or the PCR method that was conducted and interpreted. The flow and timing described any patients who did not receive the index tests or reference standard. Each question was answered with a “yes,” “no,” or “unclear” response. The results of the QUADAS assessment for all included studies were then summarized in the methodological quality graph and summary created by Review Manager.

**Publication bias.** Publication bias is the publication of studies due to the statistical significance of the results26, which can lead to overestimated effect sizes and the dissemination of false-positive results27. The publication bias was assessed by visual inspection of funnel plot asymmetry (the asymmetrical distribution of the included studies in the graph between the OR and SE (logOR)). The publication bias was also assessed with Egger's test. Both tests aimed to determine small-study effects leading to more or less beneficial summaries of OR estimates27.

**Results**

**Characteristics of the included studies.** The search retrieved 1,340 records. After removing 144 duplicates, 1,196 records were left for the title and abstract screening. Title and abstract screening resulted in the exclusion of 946 records. The full texts of 250 articles were assessed for their eligibility, and 231 of these were excluded with tags indicating the reason for exclusion. The most common reason for exclusion was no report of RDT in their articles. Other reasons for exclusion are shown in Fig. 1. As a result, 19 articles were included in the present study26–44. Further searches on the references of the selected publications which passed the inclusion criteria and Google Scholar search resulted in the inclusion of 9 additional articles45,46–53. Overall, 28 articles were selected, extracted, and analysed.

Among the 28 articles included in the present study, 3 reported mixed infections by RDT type 2 (pf-HRP2/pan-aldolase) and microscopy47,49,13, 1 by RDT type 3 (pf-HRP2/pan-pLDH) and microscopy47,21,27,33,38,39,41,44,46,7, 3 by type 4 (Pf-LDH/pan-pLDH) and microscopy47,3, 1 by RDT type 5 (Pf/Pv-LDH)48, and 9 by RDT type 6 (pf-HRP2/Pv-pLDH)47,21,26,36,42,43,46,48–50. Among 27 articles included in the present study, 1 reported mixed infections by RDT type 2 and PCR32. 5 on RDTs type 3 and PCR21,26,33,45,49 and 5 by RDT type 6 and PCR21,26,29,37,43. Most of the included studies (8/26, 30.8%) were conducted in Ethiopia27,30,31,36,42,45,48,50, India (3/26, 11.5%)32,39,49, and Kuwait (2/26, 7.7%)34,46. Additional data are shown in Table 1.

**WHO product testing of malaria RDTs.** The WHO product testing of malaria RDTs began in 200852. All companies manufacturing malaria RDTs under the ISO-13485 Quality System Standard were invited to submit up to three tests for evaluation52. The results of the WHO product testing of malaria RDTs are demonstrated in Table 2. RDTs from the eight studies30,34,36,40,41,46,49,51 were not subject to the WHO product testing program as Table 1.

**Methodological quality of the included studies.** The methodology and reporting of the selected studies varied highly (Fig. 2; Supplementary Fig. 1). All 28 included studies had cross-sectional designs. Most of the included studies (25/28, 89.3%) used a consecutive or random sample of patients. Two studies did not enrol a
consecutive or random sample of patients21,36. In another study19, the sampling method for participants enrolled was unclear. Microscopic examination was used as the reference standard in 24 studies. PCR was used as a refer-

Figure 1. Study flow diagram.
| No. (Ref) | Author                  | Study area (years of this survey) | Participants (N) | Malaria positives | Mono infections | Mixed infections | Method | Manufacturer | Antigen | Type | Molecular techniques | PCR method (gene) | Malaria positive | Mono infections | Mixed infections |
|----------|-------------------------|----------------------------------|------------------|-------------------|----------------|----------------|--------|--------------|---------|------|----------------------|------------------|----------------|----------------|-----------------|
| 1. Ref | Ali et al., 2011        | Bangladesh (2009–2014)           | 189              | 186               | 3              | Thick and shreek blood films | Ouchter (PVDF; CIBA, Inc., USA) | p1-HER2/Pv-pLDH | 3          | 178  | 179  | Real-time PCR (18S rRNA) | 106              | 106            | 8              |
| 2. Ref | Ahmed et al., 2016      | Ethiopia (2014)                  | 552              | 140               | 8              | Thick and shreek blood films | Cartellet (Access Bio, Inc., USA) | p1-HER2/Pv-pLDH | 3          | 738  | 736  | ND                | ND               | ND             | ND             |
| 3. Ref | Berton et al., 2008     | Equatorial Guinea (2003)         | 655              | 380               | 8              | Thick and shreek blood films | Malaria: Malara 4 antigen (Test cassette) (359 in Indonesia, Germany) | p1-HER2/Pv-pLDH | 3          | 927  | 927  | Some nested multiplex PCR (18S rRNA) | 797              | 772            | 10             |
| 4. Ref | Berne et al., 2004      | Gabon (2012)                     | 94               | 8                 | 1              | Thick and shreek blood films | QD ISEHH: Malaria Ag, PV (Standard Diagnostics Inc., Republic of Korea) | p1-HER2/Pv-pLDH | 3          | 100  | 100  | 1*                | ND               | ND             | ND             |
| 5. Ref | Chao et al., 2015       | Eqatropic Guinea (2007–2014)     | 226              | 216               | 2              | Thick and shreek blood films | Cartellet: Malara FF (BioMedicals, Inc., USA) | p1-HER2/Pv-pLDH | 3          | 200  | 200  | 2                | ND               | ND             | ND             |
| 6. Ref | Edmonson et al., 2005   | Ethiopia (2008–2014)             | 120              | 90                | 2              | Thick and shreek blood films | Test Biopatry: Malara Antigen (PVDF; CareStart (Pty Ltd, Republic of Korea)) | p1-HER2/Pv-pLDH | 3          | 100  | 100  | 1*                | ND               | ND             | ND             |
| 7. Ref | Elhajer et al., 2013    | Senegal (2013)                   | 100              | 98                | 2              | Thick and shreek blood films | Carlett Malara: HRP-2 (BioMedicals, Inc., USA) | p1-HER2/Pv-pLDH | 3          | 81   | 77   | 14               | ND               | ND             | ND             |
| 8. Ref | Eshelbore et al., 2010  | Ethiopia (2015)                  | 120              | 90                | 2              | Thick and shreek blood films | Sarriett: Malara HRP-2 (BioMedicals, Inc., USA) | p1-HER2/Pv-pLDH | 3          | 90   | 70   | 12               | ND               | ND             | ND             |
| 9. Ref | Feleke et al., 2015     | Ethiopia (2015)                  | 287              | 94                | 1              | Thick and shreek blood films | First Response: Malara Anti-pan (PVDF; CIBA, Inc., USA) | p1-HER2/Pv-pLDH | 3          | 94   | 87   | 1*                | ND               | ND             | ND             |
| 10. Ref| Gomu et al., 2015       | Ethiopia (2015)                  | 220              | 200               | 0              | Thick and shreek blood films | First Response: Malara Anti-pan (PVDF; CIBA, Inc., USA) | p1-HER2/Pv-pLDH | 3          | 216  | 216  | 0                | Quantitative real-time PCR (18S rRNA) | 900              | 900            | 90             |
| 11. Ref| Hammad et al., 2016     | Bangladesh (2015–2016)           | 189              | 186               | 3              | Thick and shreek blood films | Cartellet: Malara FF (BioMedicals, Inc., USA) | p1-HER2/Pv-pLDH | 3          | 80   | 66   | 14               | ND               | ND             | ND             |
| 12. Ref| Imwong et al., 2016     | India (2016)                     | 752              | 744               | 8              | Thick and shreek blood films | ICT Malaria Ag, PVDF/ICT (Diagnostics, Ambleside, UK) | p1-HER2/Pv-pLDH | 3          | 75   | 66   | 17               | ND               | ND             | ND             |
| 13. Ref| Iqbal et al., 2001      | Pakistan (1999–2001)             | 163              | 151               | 12             | Thick and shreek blood films | OpioHLA (Opio Diagnosics, Inc., Republic of Korea) | p1-HER2/Pv-pLDH | 3          | 150  | 150  | 12               | ND               | ND             | ND             |
| 14. Ref| Iqbal et al., 2002      | Pakistan (1999–2002)             | 211              | 207               | 4              | Thick and shreek blood films | ApoHLA (Opio Diagnosics, Inc., Republic of Korea) | p1-HER2/Pv-pLDH | 3          | 207  | 207  | 4                | ND               | ND             | ND             |
| 15. Ref| Iqbal et al., 2001      | Pakistan (2003)                  | 120              | 100               | 0              | Thick and shreek blood films | ApoHLA (Opio Diagnosics, Inc., Republic of Korea) | p1-HER2/Pv-pLDH | 3          | 100  | 100  | 0                | ND               | ND             | ND             |

Continued
Discordance between RDTs and microscopy. All 24 studies reporting on the performance of RDTs for detecting mixed infections compared to microscopy were explicitly designed for this purpose. In total, six different RDT types including RDT types 2, 3, 4, 5 and 6 were included in the analysis. One study did not report the type of RDT used in their study. Six studies used more than one RDT type/brand in their studies. Four studies reported mixed infections by RDT, but no mixed infections were reported by microscopy. The results of an individual study demonstrated that 18 studies in total had identical results for RDT type 2 and microscopy. Six studies gave identical results for RDT type 3 and microscopy. Two studies gave identical results for RDT type 4 and microscopy. Nine studies yielded identical results for RDT type 6 and microscopy. The summary estimate of ORs...
| No. (ref.) | Authors          | Positivity | Plasmodium spp. | Microscopy | RDTs | Antigen | WHO product testing | False positive (%) Plasmodium spp. infection in clean-negative samples | Number of mixed infections | Types of mixed infections | Number of mixed infections | Types of mixed infections |
|-----------|------------------|------------|-----------------|------------|------|---------|---------------------|-------------------------------------------------|--------------------------|------------------------|--------------------------|----------------------------|
| 1. Ref.26 | Alam et al., 2011 | 3          | P. falciparum/P. vivax | Venipuncture | Onsite Pf/Pv (CTK Biotech Inc., USA) | pf-HRP2/ Pv-pLDH | Round 2 | 0                  | 5  | P. falciparum/P. vivax | 8  | P. falciparum/P. vivax (8) |
| 2. Ref.27 | Ashton et al., 2010 | 9          | P. falciparum/P. vivax | Finger prick | CareStart (Access Bio, Inc., USA) | pf-HRP2/ pan-pLDH | Round 1 | 3.0               | 320 | P. falciparum/P. vivax | 336 | P. falciparum/P. vivax |
| 3. Ref.28 | Berzosa et al., 2018 | 0          | P. falciparum/P. malariae | Finger prick | NADAL Malaria 4 species test (Test cassette) (Nal von Minden, Germany) | pf-HRP2/ pan-pLDH | Not found in WHO product testing records | Not found in WHO product testing records | 212 | Not specified | 15 | Not specified |
| 4. Ref.29 | Bouyou et al., 2014 | 1          | P. falciparum/P. vivax | Venipuncture | SD BIOLINE Malaria Ag /Pv Panel (Standard Diagnostics Inc., Republic of Korea) | pf-HRP2/pan-pLDH | Round 4 | 1.3               | 1  | P. falciparum/P. malariae | 20 | P. falciparum/P. vivax |
| 5. Ref.30 | Chanie et al., 2011 | 2          | P. falciparum/P. vivax | Finger prick | CareStart Malaria Pf/Pv Combo test (Access Bio, Inc., USA) | pf-HRP2/ Pv-pLDH | Round 2 | 0                  | 4  | P. falciparum/P. vivax | 12 | P. falciparum/P. vivax |
| 6. Ref.31 | Edwards et al., 2015 | 2          | P. falciparum/P. vivax | Venipuncture | SD BIOLINE Malaria Ag Pf/Pv Pan (Standard Diagnostics Inc., Republic of Korea) | pf-HRP2/ Pv-pLDH | Round 4 | 2.8               | 2  | P. falciparum/P. vivax | 20 | P. falciparum/P. vivax |
| 7. Ref.32 | Ehtesham et al., 2015 | 2          | P. falciparum/P. vivax | Venipuncture | First Response Malaria Antigen pLDH/HRP2 Combo (Premier Medical, India) | pf-HRP2/pan-pLDH | Round 2 | 0                  | 14 | P. falciparum/P. vivax | 12 | P. falciparum/P. vivax |
| 8. Ref.33 | Endeshaw et al., 2010 | 84         | P. falciparum/P. vivax | Finger prick | Parascreen Pan/Pf (Zephyr Biomedical systems, India) | pf-HRP2/ pan-pLDH | Not assessed | Not assessed | 75 | P. falciparum/P. vivax | 75 | P. falciparum/P. vivax |
| 9. Ref.34 | Feleke et al., 2017 | 5          | P. falciparum/P. vivax | Venipuncture | CareStart Malaria HRP2/pLDH (Pf/Pv) Combo (Access Bio, Inc., USA) | pf-HRP2/pan-pLDH | Round 5 | 0.4               | 5  | P. falciparum/P. vivax | 5  | P. falciparum/P. vivax |

Continued
| No. (ref.) | Authors | Microscopy | RDTs | PCR results |
|-----------|---------|------------|------|-------------|
| 10. Ref.45 | Getnet et al., 2015 | Microscopy R DTs PCR results |
| 11. Ref.32 | Haanshuus et al., 2016 | |
| 12. Ref.33 | Imwong et al., 2015 | |
| 13. Ref.34 | Iqbal et al., 2001 | |
| 14. Ref.46 | Iqbal et al., 2002 | |
| 15. Ref.35 | Jahan et al., 2019 | |
| 16. Ref.47 | Khorashad et al., 2014 | |
| 17. Ref.36 | Kim et al., 2008 | |
| 18. Ref.37 | Li et al., 2016 | |
| 19. Ref.49 | Meena et al., 2009 | |

**Microscopy**
- CareStart Malaria HRP2/ pLDH (Pf/PAN) Combo (Access Bio, Inc., USA)
- ParaHIT-Total Ver. 1.0 Device 55K204-10 (Span Diagnostics Ltd, India)
- 55IC204-10 (Span Diagnostics Ltd, India)
- SD BIOLINE Malaria Ag Pf/Pv POCT (Standard Diagnostics Inc., Republic of Korea)
- First response Malaria Ag, pLDH/HRP2 Combo Card test kit (Premier Medical Corporation Ltd.)
- OptiMAL (Biorad, France)
- Venipuncture
- SD BIOLINE Malaria Ag Pf/Pv POCT (Standard Diagnostics Inc., Republic of Korea)
- Venipuncture
- Malaria P/I (p.d/pv) POCT kits (InTec Products Inc., China)

**RDTs**
- pf-HRP2/pan-pLDH
- pf-HRP2/pan-pLDH
- pf-HRP2/pan-pLDH
- pf-HRP2/pan-pLDH
- pf-HRP2/pan-pLDH
- pf-HRP2/pan-pLDH
- pf-HRP2/pan-pLDH

**PCR results**
- P. falciparum/P. vivax (5)
- P. falciparum/P. vivax (27)
- P. falciparum/P. vivax (56)
- P. falciparum/P. vivax (5)
- P. falciparum/P. vivax (56)
- P. falciparum/P. vivax (56)
- P. falciparum/P. vivax (56)
- P. falciparum/P. vivax (56)
- P. falciparum/P. vivax (56)
- P. falciparum/P. vivax (56)
- P. falciparum/P. vivax (56)
- P. falciparum/P. vivax (56)

**Blood collection methods**
- Manufacturers Antigen WHO product testing Number of mixed infections Types of mixed infections Number of mixed infections Types of mixed infections
### Table 2. Characteristic of Plasmodium mixed infections.

| No. (ref.) | Authors                | Microscopy                      | RDTs                              | False positive (%) | Number of mixed infections | Types of mixed infections | Types of mixed infections |
|------------|------------------------|---------------------------------|-----------------------------------|--------------------|---------------------------|--------------------------|--------------------------|
| 20. Ref.<sup>46</sup> | Mehlotra et al., 2019   | 0                               | Finger prick                     | pf-HRP2/pan-pLDH   | Round 5                   | 0                        | 372                      | 24  |
|            |                        |                                 | SD BIOLINE Malaria Ag P/P Pan (Standard Diagnostics Inc., Republic of Korea) |                    |                           |                          |                          |     |
| 21. Ref.<sup>38</sup> | Mogens et al., 2012    | 6                               | Finger prick                     | pf-HRP2/pan-pLDH   | Round 1                   | 3.0                      | 26                       | 0   |
|            |                        |                                 | CareStart Malaria HRP2/pan-LDH (P/P) (Access Bio, Inc., USA) |                    |                           |                          |                          |     |
| 22. Ref.<sup>39</sup> | Ranjan et al., 2016    | 0                               | Venipuncture                      | Not reported       | Not reported              | –                        | –                        | 3   |
|            |                        |                                 |                                   |                    |                           |                          |                          |     |
| 23. Ref.<sup>40</sup> | Ratnasari et al., 2008 | 22                              | Not specified                     | pf-HRP2/pan-pLDH   | Not assessed              | Not assessed             | 22                       | 0   |
|            |                        |                                 | Rapid One-Step Malaria test (Arista Biologicals Inc., USA) |                    |                           |                          |                          |     |
| 24. Ref.<sup>41</sup> | Richter et al., 2004   | 4                               | Not specified                     | pf-HRP2/pan-pLDH   | Not assessed              | Not assessed             | 4                        | 0   |
|            |                        |                                 | The Now Malaria test (Binax, Inc., USA) |                    |                           |                          |                          |     |
| 25. Ref.<sup>30</sup> | Sharew et al., 2009    | 10                              | Not specified                     | pf-HRP2/pan-pLDH   | Round 2                   | 0.5                      | 10                       | 0   |
|            |                        |                                 | CareStart Malaria P/P Pv Combo test (Access Bio, Inc., USA) |                    |                           |                          |                          |     |
| 26. Ref.<sup>51</sup> | van den Broek et al., 2006 | 7                             | Finger prick                      | Pf-LDH/pan-pLDH    | Not assessed              | Not assessed             | 7                        | 0   |
|            |                        |                                 | Optimal-IT (Diamed AG, Switzerland) |                    |                           |                          |                          |     |
| 27. Ref.<sup>42</sup> | Woyessa et al., 2013   | 5                               | Not specified                     | pf-HRP2/pan-pLDH   | Round 4                   | 0                        | 14                       | 0   |
|            |                        |                                 | CareStart Malaria P/P Pv combo test (Access Bio, Inc., USA) |                    |                           |                          |                          |     |
| 28. Ref.<sup>43</sup> | Yan et al., 2013       | 11                              | Finger prick                      | pf-HRP2/pan-pLDH   | Round 1                   | 0                        | 33                       | 0   |
|            |                        |                                 | Wondfo One Step Malaria HRP2/pan-LDH (P/P) Test (Pan) |                    |                           |                          |                          |     |
|            |                        |                                 | Malaria P/P Pv test device (Tysolpharm Co., Limited, UK) | pf-HRP2/pan-pLDH   | Not found in WHO product testing records | Not found in WHO product testing records | 8 | 0 | 21 |

between type 2 RDTs and microscopy to detect mixed infections ranged from 1.18 to 51.1. Based on the analysis of three included studies, the summary estimate of ORs between type 2 RDTs and microscopy was 4.33 (95% CI 0.24–79.8, p = 0.32, I² = 96%). The summary estimate of ORs between type 3 RDTs and microscopy to detect mixed infections based on the analysis of 13 included studies was 8.46 (95% CI 2.75–26.1, p = 0.0002, I² = 96%). When the four studies that reported mixed infections based on the analysis of 13 included studies was 4.02 (95% CI 1.46–11.12, p = 0.007, I² = 95%) (Supplementary file 1). The summary estimate of ORs between type 4 RDTs and microscopy to detect mixed infections based on the analysis of three included studies, was 0.99 (95% CI 0.48–2.04, p = 0.97, I² = 8%). The summary estimate of ORs between type 5 RDTs and
Figure 2. Methodological quality of the included studies.

| Study or Subgroup | RDT Events | Microscopy Events | Odds Ratio M-H, Random, 95% CI | Odds Ratio M-H, Random, 95% CI |
|-------------------|------------|-------------------|-------------------------------|-------------------------------|
| Type 2            |            |                   |                               |                               |
| Ashton et al., 2010 (CIT-Combi) | 338 | 737 | 9 | 552 | 3.5% | 51.11 [28.04, 100.33] | 1.16 [0.28, 4.95] |
| Richter et al., 2004 (The Malaria test) | 4 | 59 | 4 | 69 | 3.1% | 1.16 [0.28, 4.95] |
| van den Broek et al., 2009 (New Malaria ICT) | 8 | 134 | 7 | 140 | 3.4% | 1.21 [0.43, 3.42] |
| **Subtotal (95% CI)** | 530 | 761 | 10.6% | 4.33 [1.82, 9.81] |
| **Total events** | 350 | 20 | | |
| Heterogeneity: Tau² = 0.32; CH² = 49.69; df = 2 (P < 0.0001), I² = 95% |
| Test for overall effect: Z = 0.66 (P = 0.52) |

Figure 3. Discordance between RDTs and microscopy.
microscopy to detect mixed infections based on the analysis of one included study was 0.87 (95% CI 0.46–1.65).

The summary estimate of ORs between type 6 RDTs and microscopy to detect mixed infections based on the analysis of nine included studies was 1.07 (95% CI 0.74–1.55, p = 0.71, I² = 0%). Overall, the significant summary estimate of ORs between all types of RDTs and microscopy to detect mixed infections was found (OR = 3.33, 95% CI 1.66–6.68, p = 0.009, I² = 94%).

The subgroup analysis of blood collection methods for microscopy was performed using 18 included studies. The results demonstrated that no subgroup difference (p = 0.55) was found among studies using blood from the finger prick method and those using blood from venipuncture. The summary estimate of ORs between all types of RDTs compared to those performing microscopy using blood from the finger prick method to detect mixed infections was significantly different among 11 studies (OR = 4.41, 95% CI 1.72–11.29, p = 0.002). The summary estimate of ORs between RDTs and microscopy using blood from the venipuncture method was significantly different among seven studies (OR = 3.04, 95% CI 1.44–6.43, p = 0.004) (Fig. 4).

Discordance between RDTs and PCR. Overall, 12 studies reported on mixed infections detected by both RDTs and PCR, as shown in Fig. 5. Among 12 studies, three different types of RDTs were reported including RDT types 2, 3, and 6. The summary estimate of ORs between type 2 RDTs and PCR to detect mixed infections was 8.21 (95% CI 4.51–15.0). The summary estimate of ORs between type 3 RDTs and PCR based on the analysis of six included studies to detect mixed infections was 4.05 (95% CI 0.73–7.84, p = 0.07, I² = 97%). The summary estimate of ORs between type 6 RDTs and PCR based on the analysis of five included studies to detect mixed infections was 0.42 (95% CI 0.26–0.68, p = 0.0005, I² = 0%). Another study with no description on the type of RDT showed that the summary estimate of ORs between RDTs and PCR to detect mixed infections was 0.84 (95% CI 0.51–1.27). Overall, the summary estimate of ORs between all types of RDTs and PCR to detect mixed infections was 1.17 (95% CI 1.54–2.53, p = 0.42, I² = 96%). The subgroup analysis of detection of Plasmodium mixed-species infections between RDT and PCR found that the summary estimate of ORs between RDT and PCR was comparable in P. falciparum mixed infections with P. vivax (OR = 0.81, 95% CI 0.51–1.27, p = 0.36, I² = 51%) and in P. falciparum/P. vivax and P. falciparum mixed infections with other Plasmodium spp. (OR: 6.96, 95% CI 1.50–32.4, p = 0.01, I²: 99%) (Fig. 6).
Figure 5. Discordance between RDTs and PCR.

| Study or Subgroup | RDT | PCR | Odds Ratio |
|-------------------|-----|-----|------------|
|                  | Events | Total | Total | Weight | M-H, Random, 95% CI |
| 2.1.1 Type 2     | Haanshuus et al., 2016 | 27 | 75 | 30 | 468 | 7.0% | 8.21 (4.51, 14.95) |
|                  | Subtotal (95% CI) | 75 | 468 | 7.0% | 8.21 (4.51, 14.95) |
|                  | Total events | 27 | 30 |   |   |
|                  | Heterogeneity: Not applicable |
|                  | Test for overall effect: Z = 6.89 (P < 0.00001) |
| 2.1.2 Type 3     | Berzosa et al., 2016 | 212 | 761 | 15 | 787 | 7.1% | 19.87 (11.64, 33.93) |
|                  | Edhesham et al., 2015 (CareStart Malaria) | 12 | 80 | 12 | 100 | 6.9% | 1.13 (0.48, 2.88) |
|                  | Edhesham et al., 2015 (First Response) | 14 | 91 | 12 | 100 | 6.9% | 1.33 (0.56, 3.09) |
|                  | Getnet et al., 2015 (CareStart PfPan) | 14 | 80 | 6 | 116 | 6.8% | 3.89 (1.43, 10.61) |
|                  | Inwong et al., 2015 | 8 | 224 | 56 | 988 | 6.9% | 0.62 (0.29, 1.31) |
|                  | Mehlora et al., 2019 | 372 | 461 | 24 | 559 | 7.1% | 93.17 (58.24, 149.05) |
|                  | Yan et al., 2013 (One Step Malaria PfPan test) | 33 | 93 | 21 | 113 | 7.0% | 2.41 (1.27, 4.55) |
|                  | Subtotal (95% CI) | 1800 | 2763 | 48.0% | 4.05 (0.88, 18.78) |
|                  | Total events | 665 | 146 |   |   |
|                  | Heterogeneity: Tau² = 4.14; Chi² = 214.89, df = 6 (P < 0.00001); I² = 97% |
|                  | Test for overall effect: Z = 1.79 (P = 0.07) |
| 2.1.4 Type 6     | Alam et al., 2011 (Falcivax P f) | 5 | 170 | 0 | 108 | 6.8% | 0.65 (0.21, 2.03) |
|                  | Alam et al., 2011 (Onsite PfPan) | 2 | 101 | 0 | 88 | 6.2% | 0.24 (0.05, 1.14) |
|                  | Edwards et al., 2015 | 2 | 103 | 20 | 174 | 6.3% | 0.15 (0.03, 0.67) |
|                  | Edhesham et al., 2015 (CareStart PfPf Combo) | 7 | 94 | 12 | 100 | 6.8% | 0.59 (0.22, 1.57) |
|                  | Li et al., 2016 | 1 | 61 | 3 | 69 | 5.4% | 0.37 (0.04, 3.62) |
|                  | Yan et al., 2013 (Malaria PfPf test device) | 8 | 90 | 21 | 113 | 6.9% | 0.43 (0.18, 1.02) |
|                  | Subtotal (95% CI) | 717 | 832 | 38.2% | 0.42 (0.26, 0.68) |
|                  | Total events | 25 | 72 |   |   |
|                  | Heterogeneity: Tau² = 0.00; Chi² = 3.45, df = 5 (P = 0.63); I² = 0% |
|                  | Test for overall effect: Z = 3.50 (P = 0.0005) |
| 2.1.5 Not specified | Ranjan P. and Ghosh U. | 3 | 92 | 3 | 78 | 6.1% | 0.04 (0.01, 0.17) |
|                  | Subtotal (95% CI) | 92 | 78 | 6.1% | 0.04 (0.01, 0.17) |
|                  | Total events | 3 | 3 |   |   |
|                  | Heterogeneity: Not applicable |
|                  | Test for overall effect: Z = 0.21 (P = 0.84) |
|                  | Total events | 720 | 261 |   |   |
|                  | Heterogeneity: Tau² = 4.68; Chi² = 341.92, df = 14 (P < 0.00001); I² = 96% |
|                  | Test for overall effect: Z = 0.80 (P = 0.42) |
|                  | Test for subarous effect: Ch² = 59.20, df = 3 (P < 0.00001); I² = 94.9% |

Figure 6. The subgroup analysis of detection of Plasmodium mixed-species infections between RDT and PCR.
Publication bias. Visual inspection of the funnel plots demonstrated no publications bias found because there was a symmetrical distribution of the included studies (geometric shapes) in the graph between the OR and SE (logOR) (Fig. 7). The publication bias was further assessed with Egger’s test. Egger’s test showed no publication bias due to the small-study effects found (p-value = 0.166) (Table S2). Therefore, the summary estimates of ORs in the present meta-analysis were not confounded by publication bias of the included studies.

Discussion
This is the first study to summarize the available data on the discrepancy between RDTs and two gold/reference standards for the detection of malaria mixed infections. The summary ORs of discrepancies of RDT types 2, 3, 4, 5, and 6 in detecting malaria mixed infections compared to microscopy were 4.33, 8.46, 0.99, 0.87, and 1.07, respectively. Even though the overall summary estimate of ORs was significantly observed, subgroup analysis of RDT types demonstrated that only RDT type 3 could detect a significantly higher proportion of Plasmodium mixed infections than the microscopic method. Among the 8 studies conducted in Ethiopia27,30,31,38,42,45,48,50 only a study by Ashton et al.27 revealed a considerable difference in the proportion of mixed infections detected by RDT types 2 and 3 compared with microscopy. From 297 blood samples of *P. falciparum* mono-infection confirmed by the microscopy, 213 (213/297: 71.7%), 224 (224/297: 75.4%), and 223 (223/297: 75.1%) samples were interpreted as mixed infections by CareStart (AccessBio, USA), ICT Combo (ICT Diagnostics, South Africa), and ParaScreen (Zephyr Biomedicals, India), respectively. The remaining studies conducted in Ethiopia had identical numbers of mixed infections in 2 studies31,50 and high numbers of mixed infections in three studies38,42,45, another study conducted in Ethiopia demonstrated more mixed infections detected by microscopy (84 cases) than by RDT (75 cases)44. Another important difference in the proportion of mixed infections detected by RDT type 3 compared with microscopy was also demonstrated in the study conducted in Madagascar by Berzosa et al. because 0.87% of blood samples with confirmed *P. falciparum* mixed infections by PCR were false positive for *Plasmodium* mixed infections by RDT type 3 (212 cases, 12.3%)28.

The high proportion of mixed infections detected by RDT types 2 and 3 compared with microscopy reported in the included studies by Ashton et al., Mehlotra et al., and by Berzosa et al. may be due to the consistent false positive Pan-pLDH test lines among *P. falciparum* samples at high parasite densities, as reported in RDTs targeting Pv-pLDH23. A high parasite density of *P. falciparum* can induce positivity of the pLDH band on RDTs, giving false positives of non-falciparum species29. The false positive on Pan-pLDH test lines among *P. falciparum* samples at high parasite densities may be possible to use as the detection limit of the SD Bioline Malaria Ag Pf/ Pan RDT used in the study by Mehlotra et al. because the mean parasitaemia level in samples that were positive for both the PfHRP2 and pan-pLDH test bands44. In addition, an important difference in the proportion of mixed infections detected by RDT type 3 compared with microscopy was also demonstrated in the study conducted in Madagascar by Berzosa et al. because 0.87% of blood samples with confirmed *P. falciparum* mixed infections by PCR were false positive for *Plasmodium* mixed infections by RDT type 3 (212 cases, 12.3%)28.

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of the mixed infection that was too low to be seen by the microscopists but with sufficient parasite antigen to yield RDT positivity57. The meta-analysis of RDTs and microscopy had no significant discrepancy among RDTs type 2, 4, and 6. In this analysis, the summary results of RDT type 5 performed by Iqbal et al. and RDTs performed by Ranjan and Ghoshal could not be interpreted because there were a small number of studies for subgroup analysis. Overall, the evidence was strong for RDT types 3 and 6 mainly because a large number of studies were available for inclusion. However, the summary estimate of RDT type 3 demonstrated high heterogeneity among the included studies (I² = 96%) when compared to those of RDT type 6 (I² = 0%). In this study, more than half of the studies (n = 18) relied solely on microscopy as the gold/reference standard for Plasmodium species identification. Therefore, the discordant results between RDTs and microscopy demonstrated in the present study might be due to the imperfect nature of the gold/reference standard because mixed infections with P. falciparum could be missed by microscopy. Because of these results, RDT types 2 and 3 could rectify the diagnosis of P. falciparum in mixed-species infections that might be missed by the microscopy method. These results supported that the selection of the most appropriate RDTs relative to malaria epidemiology and are very crucial to differentiate mixed infections because the identification of Plasmodium mixed-species infections would facilitate appropriate treatment with artemisinin-based combination therapies (ACTs), which could eliminate any mixed infection even if mixed infections were not detected by the gold/reference standard, the microscopy method56.

Recently, the sensitivity and specificity for the detection and identification of malarial parasites have been improved using the Nested-PCR method, which amplifies the 18S rRNA gene57. It has been proven to be more sensitive and accurate than routine diagnostic microscopy and provides the advantage of a higher proportion of detection in cases of mixed-species infections57. In the present study, 12 included studies used PCR as a reference standard for Plasmodium species identifications. The discrepancy between RDT type 3 and PCR (OR = 4.05) appeared to be heavily influenced by the included studies by Beraza et al. and Mehlotra et al., in which the individual ORs were extremely high (19.9 and 93.2, respectively). This affirms that when compared with using PCR as the gold/reference standard, the high discrepancy between RDT type 3 targeting Pf-HRP2 and pan-pLDH leads to incorrect interpretation of mixed infections by RDTs, as we discussed earlier in the discrepancy of RDT and microscopy. The false positive results of RDTs when detecting mixed infections may be associated with decreased age because of the high prevalence of malaria in children, particularly children under 5 years of age, who are likely to develop severe malaria with high parasitaemia58,59. The present meta-analysis demonstrated that the significant discordance between RDTs and PCR was found in studies using RDT type 6, which detects the Pf-HRP2/Pv-pLDH antigen of malaria parasites. RDT type 6 could detect a lower proportion of Plasmodium mixed infections than the PCR reference method. This finding was similar to three previous studies21,60,61. Therefore, the lower proportion of Plasmodium mixed infections detected by RDT type 6 than by PCR demonstrated in the present study might be due to the lower sensitivity and specificity of RDTs than of PCR methods. In practice, PCR methods have a higher sensitivity (approximately 0.0001 parasites/µL) than RDT (approximately 100 parasites/µL) and microscopy (approximately 50–500 parasites/µL), which allows for the detection of Plasmodium mixed infections at a low parasite density, which are routinely missed in microscopy56. The subgroup analysis of Plasmodium mixed-species infections as reported by the 6 included studies demonstrated that a comparable proportion detected P. falciparum mixed infections with P. vivax between RDTs and PCR, while there was a significant difference in the proportion that detected P. falciparum/P. vivax and P. falciparum mixed infections with other Plasmodium species. This subgroup analysis suggested that RDTs had identical results with PCR in detecting P. falciparum and P. vivax mixed infections. In contrast, RDTs had discordant results with PCR in detecting P. falciparum mixed infections with other Plasmodium species. Nevertheless, these results should be further confirmed by full experimental studies.

The present study had limitations. First, RDTs targeting HRP-2 and pan-pLDH or RDTs targeting HRP-2 and pan-aldolase are likely to be positive in P. falciparum mono-infections or mixed-species infections. Regarding this limitation of the RDTs in the included studies, the summary estimates of ORs between RDT types 2 and 3 and microscopy need to be carefully interpreted. Second, the overall evidence of the analysis between RDTs and PCR was weak, mainly because few studies were available for inclusion. Second, the lower sensitivity and specificity of RDTs than those of PCR was due to the limits of detection. The WHO has suggested that the clinical sensitivity of RDTs is highly dependent on conditions including the level of parasite density and the subset of any population, such as young children or pregnant women; thus, the interpretation of RDTs must be carefully interpreted55. Third, the sensitivity and specificity of RDTs compared to the gold standard could not be calculated due to data on individual patient were lacking and the data on whether patients who gave positive results for RDT were the same patients who gave positive results for the gold/reference standard or not, as most of the included studies report the number of positive separately between RDTs and microscopy/PCR. Fourth, some eligible studies might have been missed through the search strategy. However, the additional search of reference lists of the included studies and searches of other sources such as Google search and Google Scholar, and performing extensive searching of reference lists and searching other sources with broad search terms, helped to reduce this limitation by further increasing the number of included studies. Fifth, the study aimed to clarify what proportion of Plasmodium mixed-infections could not be confirmed by a positive RDT result, and the proportion of Plasmodium mixed infections were often not the primary target of studies, which led to a low number of studies that were focused on mixed infections. In light of these, although the current data are still suggestive of high discrepancies of RDT type 3 for detecting Plasmodium mixed infections in comparison to microscopy and of RDT type 6 for detecting Plasmodium mixed infections in comparison to PCR methods, they provided a critical advantage on malaria treatment in resource-limited settings in which the results of microscopy could not be obtained. Further studies focused on the diagnosis of Plasmodium mixed-species infections by RDTs are needed to provide a better understanding of the performance of RDTs, guide the development of an improved diagnostic
test for *Plasmodium* mixed infections, and facilitate the appropriate treatment of patients with ACTs. This will help with the elimination of malaria in endemic and non-endemic areas where laboratory capacity is limited.

**Conclusion**

In conclusion, the present study suggested that malaria RDTs showed some discordant results with microscopy and PCR. The selection interpretation of RDTs can facilitate a better diagnosis of *Plasmodium* mixed-species infections and appropriate treatment of malaria patients in endemic and non-endemic settings.

**Consent for publication.** All authors have read the manuscript and consent to its publication.

**Data availability**

The datasets used during the current study are available without restriction and demonstrated in the present manuscript and additional files.

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Author contributions
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Competing interests
The authors declare no competing interests.

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