Assessment of Microscopic Detection of Malaria with Nested Polymerase Chain Reaction in War-Torn Federally Administered Tribal Areas of Pakistan

Muhammad Faisal Nadeem1 · Aamer Ali Khattak2 · Nadia Zeeshan1 · Usman Ayub Awan2 · Adnan Yaqoob1

Received: 2 September 2020 / Accepted: 13 March 2021 / Published online: 11 April 2021
© Witold Stefański Institute of Parasitology, Polish Academy of Sciences 2021

Abstract

Introduction Diagnostic accuracy of malaria is critical for early treatment, control, and elimination of malaria, especially in war-affected malaria-endemic areas. Microscopic detection of Plasmodium species has been the gold standard in remote malaria-endemic regions. However, the diagnostic accuracy is still questioned, especially in discriminating mixed and sub-microscopic parasitic levels. This study was designed to evaluate the diagnostic performance of microscopic examination against nested PCR analysis in war-torn malaria-endemic Federally Administered Tribal Areas (FATA) of Pakistan.

Methods Venous blood samples were collected from symptomatic patients for microscopic examination and nested PCR analysis from January 2016–December 2016 from five Agencies (Bajaur, Mohmand, Khyber, Orakzai and Kurram Agency) and four Frontier Regions (Peshawar, Kohat, Bannu, and Dera Ismail Khan Frontier Region) of FATA. Malaria-positive isolates were confirmed by nested PCR (targeting Plasmodium small subunit ribosomal ribonucleic acid (ssrRNA) genes) for speciation.

Results Among enrolled participants, 762 were found positive for malaria parasite on microscopic examination of the blood film. Plasmodium vivax was found in 623, Plasmodium falciparum in 132 and 7 were diagnosed with mixed infection (P. vivax and P. falciparum coinfection). Nested PCR detected Plasmodium infection in 679 samples (523 P. vivax, 121 P. falciparum, and 35 mixed infections). Compared with microscopy, the sensitivity of nested PCR was 98.94%, and specificity was 98.27%, while the sensitivity and specificity of slide microscopy 89.34% and 87.99% respectively.

Conclusion The conventional microscopy method has low sensitivity to detect the mixed infection as compared to nested PCR. High sensitivity and specificity observed in nested PCR make this molecular tool a useful technique for monitoring, controlling, and eliminating malaria-endemic regions.

Keywords Comparison · Diagnostic method · Malaria · Nested PCR · FATA · Pakistan

Introduction

The World Health Organization malaria report showed an estimated 216 million malaria cases worldwide, with 0.45 million deaths reported in 2017 from 91 countries. It is the second most reported disease in Pakistan, affecting 4.5 million individuals with 33 reported and 1100 estimated deaths in 2016 [1]. More than 80% of nationwide malaria incidences previously reported were from the war-torn Federally Administered Tribal Area (FATA) [2]. FATA has the highest annual parasite incidence (17.64) in the country (followed by Balochistan and Khyber Pakhtunkhwa provinces) [3], and about 0.1 million cases were screened for malaria in 2016 [4]. Among many factors that influence malaria emergence in this region are political instability, the war against terrorism, mass population displacement within the country (IDPs) and across the border, underprivileged socioeconomic conditions, declining health infrastructure, poor diagnostic, preventive and curative services, regional tribal and sectarian strife all contributing to this huge disease burden [5]. For such remote and under-developed regions,
malaria is severe both in local and focal infections [6, 7]. *Plasmodium* five species (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*) are responsible for causing malarial infection in humans. Among the two globally predominant *Plasmodium* species, *P. vivax* is the most prevalent species in Pakistan that account for 80% of the malaria cases, while *P. falciparum* causes infection in an estimated 20% [8]. Incidence of double infections with both *P. falciparum* and *P. vivax* in FATA region has been reported 4–5% of all malaria infection that is quite high as compared to other parts of the country. In FATA region the rate of co-infection with *P. falciparum* and *P. vivax* is quite higher (4–6%) than other malaria-endemic regions of Pakistan [5]. Diagnosis and surveillance of malaria in a region where more than one species of *Plasmodium* genus are present are difficult to achieve without using a more sensitive, specific, and reliable diagnostic technique.

Even though empirical diagnosis results in overtreatment due to inaccuracy, it remains the basis for malaria diagnosis in a majority of the symptomatic patients in malaria-endemic regions, where advanced diagnostic facilities are lacking. Nevertheless, a clinical or presumptive diagnosis is the least expensive and most commonly used method that forms the basis for self-treatment in areas such as FATA [9].

A variety of diagnostic tools are available for *Plasmodium* identification and speciation, including microscopy (light and fluorescence), immuno-chromatographic techniques, nucleic acid amplification methods (PCR and isothermal amplification), and flow cytometry [10]. Dielectrophoretic and magnetophoretic approaches are also among the promising new techniques, but their sensitivity and specificity still have to be evaluated [11]. Laboratory diagnosis of malaria at the primary healthcare level in remote areas is limited to light microscopy. However, this technique has some advantages and shortcomings in proper malaria diagnosis [12]. To overcome the shortcomings of microscopy, a rapid diagnostic test (RDT) has been developed for malaria detection, which is claimed to be simple, accurate, and cost-effective diagnostic tests for malaria. Like microscopy, RDT also has limitations [13], particularly in differences in sensitivity among various kits [14].

During the last decade, the molecular diagnostic technique Polymerase Chain Reaction (PCR) (amplification of 18S rRNA genes) has gained popularity over conventional microscopic technique and RDT [15]. The PCR technique has demonstrated its high specificity and sensitivity in *Plasmodium* identification and speciation [15–18]. Besides detection and mixed-species identification [19–21], PCR has also proven its remarkable utility in follow-up studies on anti-malarial efficacy, molecular epidemiology, and drug resistance studies [7, 8, 13, 22]. PCR has been proven to be superior, but the molecular diagnostic setup is not always possible as it is expensive, time-consuming, need expertise, supervision, and resources [23]. That’s why PCR-based malaria diagnosis may not be feasible in remote rural areas such as FATA [24].

As per WHO malaria guidelines, all suspected cases must undergo laboratory diagnosis using microscopy and RDT before any therapeutic care because information regarding the effectiveness of presumptive diagnosis is inadequate. This study was conducted to evaluate the performance of nested PCR in the detection of malarial parasites in positive microscopy samples in a war-torn, remote malaria-endemic region of Pakistan.

### Materials and Methods

The study population was comprised of patients visiting healthcare facilities in five Agencies (Bajaur, Mohmand, Khyber, Orakzi, and Kurram Agencies) and four Frontier Regions (Peshawar, Kohat, Bannu, and Dera Ismail Khan Frontier Region) of FATA, Khyber Pakhtunkhwa province from January 2016 to December 2016. Ethical clearance for this study was obtained from the Institutional Review Board of the University of Gujrat, Pakistan. Inclusion criteria were malaria febrile patients irrespective of age and gender (having fever, chills, nausea, vomiting, sweats, headache, and fatigue) willing to give oral or written consent (parents or guardians for those below 18 years) to be part of the research. Individuals who were already taking anti-malarial drugs in the past 2 weeks or those who did not consent to be part of the study were excluded.

3 mL of venous blood was collected from patients in a vacutainer tube containing EDTA. Thick and thin blood films were prepared, stained with 3% Giemsa staining solution and observed under the 100x objective of the microscope at the study site [25, 26]. 50 μL of whole blood from microscopy positive patients was spotted on properly labeled 3MM Whatman filter paper, air-dried separately to avoid contamination, and were then placed in a zip lock bag with a desiccant for future analysis.

For molecular analysis, the *Plasmodium* genomic DNA was extracted from dried blood spots on the Whatman filter paper by using a previously published method [27]. PCR reagent and thermal cycler conditions were adapted from previously published research work [8]. MRA-178, 3D7/ HB3/Dd2, MRA-179, and MRA-180 were used as positive controls for *P. vivax*, *P. falciparum*, *P. malariae*, and *P. ovale*, respectively, while for the negative control, ultra-pure water was used. The PCR products were resolved by electrophoresis, stained with ethidium bromide, and visualized with ultraviolet illumination.

DAG_Stat online statistics software was used to evaluate diagnostic tests such as sensitivity, specificity, predicted
values, and 95% confidence intervals (CI). In addition, Vassar Stats tool for performing statistical computation was used to estimate Kappa coefficients [28]. The kappa values were used to classify the strength of agreement between each microscopy and nested PCR. Additionally, Standard formulae were used to calculate positive predictive values [TP/(TP + FP)] negative predicted values [TN/(FN + TN)] and test accuracy [(TP + TN)/number of all tests]. For diagnostic accuracy to avoid the risk of bias due to shortcomings in design and conduct, STARD 2015 guidelines of Reporting of Diagnostic Accuracy were followed whenever required during analysis [29].

**Results**

Among malaria suspected population, 762 people were found positive on microscopic examination of Giemsa-stained blood films. *P. vivax* parasite was found in 623 (82%), 132 (17%) were positive for *P. falciparum*, and 7 (1%) had mixed infection (a mixed infection counting for both *P. vivax* and *P. falciparum* species as positive).

All microscopy tested Plasmodium positive samples were subjected to parasitic DNA extraction to identify *Plasmodium* species using PCR. In the first round of nested PCR, the product size was 1100 bp. Agarose gel electrophoresis revealed a 205 bp amplification product for *P. falciparum*, 120 bp for *P. vivax*, 144 bp for *P. malariae* and 800 bp DNA *P. ovale* in parallel with 100 bp DNA ladder; however, none of the isolate showed *P. malariae* and *P. ovale* species, as shown in Fig. 1.

However, discordant results were observed among several malaria positive isolates, and discrepancies of *Plasmodium* species were observed between results of both techniques, which are depicted in Table 1. 523 (77%) microscopy positive malarial isolates were identified as *P. vivax* by nested PCR, 121 (18%) were confirmed as *P. falciparum*, 35 (5%) were found to harbor mixed infection and 83 samples tested negative, as shown in Fig. 2. None of the samples tested positive for *P. malariae* or *P. ovale*, Slides were not available for re-examination to resolve discrepancies, but DNA was re-extracted, and nested PCR was repeated.

To compare the agreement between microscopy and PCR, Cohen’s kappa coefficient was used, which showed a high (kappa = 0.89) agreement for the presence or absence of *Plasmodium* infection. Nevertheless, for *Plasmodium* species, the coinfection agreement was low (kappa = 0.51) because PCR identified 33 (7%) samples as mixed-species infections, which were declared mono-species infections by microscopy. The agreement between the diagnostic tools, microscopy, and PCR in diagnosis as *P. falciparum* and *P. vivax* were kappa = 0.84.

PCR Sensitivity and Specificity were calculated by using microscopy as a reference method. Sensitivity of PCR was 98.94% (95% CI 97.93–99.54) and specificity was 98.27% (95% CI 96.99–99.10). Comparatively, microscopic analysis

![Image of agarose gel electrophoresis](image_url)

**Fig. 1** Nested PCR results a genus *Plasmodium*, b *Plasmodium vivax* and c *Plasmodium falciparum* Lane 1: 100 bp ladder, Lane + C: Positive control, Lane − C: Negative control

| Plasmodium parasites detection by microscopy and nested-PCR (*n* = 762) |
|---------------------------------------------|
| **Microscopy results** | **Nested-PCR results** |
| *P. vivax* (623) | *P. vivax* (518), *P. falciparum* (19), Mixed (15), Negative (71) |
| *P. falciparum* (132) | *P. vivax* (5), *P. falciparum* (98), Mixed (18), Negative (11) |
| Mixed (7) | *P. vivax* (0), *P. falciparum* (4), Mixed (2), Negative (1) |

'n number of positive blood samples examined.
seemed to be less specific with specificity 87.99% (95% CI 85.33–90.32) and sensitivity 89.34%, (95% CI 86.93–91.45), as shown in Table 2. Test of the agreement was calculated by comparison of PCR with blood film examination. PCR showed greater specificity and sensitivity over the manual microscopic examination.

Discussion

Malaria is the second most reported disease in Pakistan, with about 98% of the population at varying risk of contracting malaria infection and about 29% living in highly endemic areas, including FATA [4]. These *Plasmodium* endemic regions of Pakistan share borders with WHO-declared malaria-endemic countries Iran and Afghanistan [5]. Malaria surveillance data has been made available to national and provincial malaria control programs by a web of diagnostic centers established by local government and international donor agencies[4]. FATA is the most neglected part of Pakistan in terms of healthcare facilities. Health Directorate is chiefly responsible for providing basic health services, but the healthcare systems have been badly affected due to constant military conflicts, political unrest in the neighborhood Afghanistan and the ongoing war against terror. [6]. Malaria is the leading cause of morbidity in the FATA region, which contributes 80% of the disease burden of malaria in Pakistan with the highest test positivity rate (17.01%) and API (17.64) [30].

Many epidemiological studies have reported a high prevalence of *P. vivax* infection, 80–90% compared to *P. falciparum* 10–20% [3]. Incidence of double infections with both *P. falciparum* and *P. vivax* in FATA region has been reported 4–5% of all malaria infection that is quite high as compared to other parts of the country [5]. However, 363 malaria diagnostic centers established in FATA for a population of about 5 million can’t meet the public health demand for malaria management. A presumptive diagnosis seems the only solution in such an area for treating malaria patients [31].

Although Giemsa-stained blood film microscopy has been recommended as a gold standard malaria diagnostic technique, in those areas where microscopy is not possible (due to unavailability of either microscope or trained staff), prompt malaria confirmation can be done using rapid diagnostic tests (RDTs). Microscopic examination has advantages such as low direct costs, sensitivity to differentiate between malaria species, and the ability to measure parasitemia level before and after treatment. However, many factors affect the quality of results generated through microscopic examination of blood films. These include parasitemia detection level per microliter of blood, species identification constraints, insufficient staff, competency level of staff, substandard or inappropriate equipment. These factors contribute towards reporting false-negative and false-positive results and discrepancies in species identification using microscopy. The predisposition to report febrile patients as parasite-positive has been reported and factors such as contaminated reusable microscope slides, staining artifacts, lack of microscopic
PCR-based methods are useful in detecting malaria parasite, of than microscopy. We recommend using PCR for the detection of malarial parasites. All performance characteristics revealed that PCR has higher sensitivity and specificity for the detection as mixed species infection than microscopy. There is a need for PCR detection as missed or misread infections by microbiologists are very common in areas where more than one plasmodium species co-exist. Higher sensitivity and specificity associated with the nested PCR, molecular detection method holds enormous promise for malaria diagnosis and can be used as effective monitoring tools for surveillance, control, and malaria elimination from a remote area such as FATA. Based on the findings, we strongly recommend using the PCR technique as the reference method in government institutes and diagnostic center to diagnose malarial parasite and verify blood film results and investigate the drug-resistant strains of plasmodium species. In epidemiological studies, to know the different plasmodium species around the country, as well as to carry out a quality control of the microscopic diagnosis should be made in different laboratories of the country.

The present study has reported high sensitivity and specificity of PCR [24, 25]. In contrast, a study reported the less sensitivity and specificity of PCR lower than the current study [26]. This difference is possibly due to PCR also has few disadvantages such as it is time-consuming, involves many steps, requires technical staff, and expensive because of the complex equipment needed to run the PCR which hinders PCR implementation as an alternative to microscopy in remote areas and low-income countries.

The results of microscopy compared with nested PCR revealed that PCR has higher sensitivity and specificity for the detection of malarial parasites. All performance characteristics such as sensitivity, specificity, positive predictive value, negative predictive value and accuracy were higher for nested PCR than microscopy. We recommend using PCR for the detection of Plasmodium infection and also for species differentiation. PCR-based methods are useful in detecting malaria parasite, but recently this molecular tool also showed its supremacy in evaluating treatment monitoring response, molecular drug resistance, and parasite insecticide resistance detection.

Conclusion

Our study reported that PCR detected Plasmodium and discriminated very well between different species when compared to microscopy. Nested PCR is very effective in detecting mixed species infection than microscopy. There is a need for PCR detection as missed or misread infections by microbiologists are very common in areas where more than one plasmodium species co-exist. Higher sensitivity and specificity associated with the nested PCR, molecular detection method holds enormous promise for malaria diagnosis and can be used as effective monitoring tools for surveillance, control, and malaria elimination from a remote area such as FATA. Based on the findings, we strongly recommend using the PCR technique as the reference method in government institutes and diagnostic center to diagnose malarial parasite and verify blood film results and investigate the drug-resistant strains of Plasmodium species. In epidemiological studies, to know the different Plasmodium species around the country, as well as to carry out a quality control of the microscopic diagnosis should be made in different laboratories of the country.

Acknowledgements The authors are thankful to Dr. Aamer Ali Kathkak, Dr. Huma Fatima, Mr. Adnan Yaqoob, and other Lab staff for cooperating in extraction, amplification of DNA, and DNA analysis.

Author contributions MFN and AAK conceived, designed the paper, and supervised the research. AAK, AY, and UAA wrote the manuscript. All the authors read and approved the manuscript.

Funding None to declare.

Availability of data material All the relevant data is available and can be provided upon request.

Declarations

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval Ethical approval is obtained from the ethical committee, Department of Medical Laboratory Technology, University of Haripur, Haripur, Khyber Pakhtunkhwa, Pakistan.

References

1. World Health Organization (2018) World Malaria Report-2017. WHO Global Malaria Programme
2. Hussain K, Shafee M, Khan N, Jan S, Tareen A, Khan M (2013) Serore prevalence of pediatric malaria in quetta balochistan Pakistan. Iran J Parasitol 8:342–347

3. World Health Organization (2015) Global technical strategy for malaria 2016–2030. World Health Organization, Geneva, p 2017

4. Directorate of Malaria Control (2016) Malaria annual report 2016. Program Management Unit Directorate of Malaria Control, Pakistan

5. Karim AM, Hussain I, Malik SK, Lee JH, Cho IH, Kim YB, Lee SH (2016) Epidemiology and clinical burden of malaria in the war-torn area, orakzai agency in Pakistan. PLoS Negl Trop Dis 10:e0004399. https://doi.org/10.1371/journal.pntd.0004399

6. Khan AQ, Ali I, Imran M, Yaseen M, Abbas SZ, Mufti FUD, (2016) Epidemiological study in FATA areas of Khyber-Pakhtunkhwa, Pakistan. Prof Med J 23:553–558. https://doi.org/10.4324/9781003007784-3

7. Khattak AA, Venkatesan M, Jacob GC, Artimovich EM, Nadeem MF, Nighat F, Hombhanje F, Mita T, Malik SA, Plowe CV (2013) Comprehensive survey of polymorphisms conferring anti-malarial resistance in Plasmodium falciparum across Pakistan. Malar J 12:300. https://doi.org/10.1186/1475-2875-12-300

8. Khattak AA, Venkatesan M, Satti HS, Yaqoob A, Strauss K, Khatoon L, Malik SA, Plowe CV (2013) Prevalence and distribution of human Plasmodium infection in Pakistan. Malar J 12:297. https://doi.org/10.1186/1475-2875-12-297

9. Ojulongbe O, Adegbosin OO, Taiwo SS, Alli OA, Olowu OA, Ojulongbe TA, Bolaji OS, Adegbosin OA (2013) Assessment of clinical diagnosis, microscopy, rapid diagnostic tests, and polymerase chain reaction in the diagnosis of Plasmodium falciparum in Nigeria. Malar Res Treat 2013:308069. https://doi.org/10.1155/2013/308069

10. Siwak N, Singh KS, Dash M, Kar S, Rani S, Rawal C, Singh R, Anvikar AR, Pande V, Das A (2018) Malaria diagnosis by PCR revealed differential distribution of mono and mixed species infections by Plasmodium falciparum and P. vivax in India. PLoS ONE 13:e0193046. https://doi.org/10.1371/journal.pone.0193046

11. Kasetsiriuk S, Buranapong J, Sriratanawich W, Kaewthamason M, Pimpin A (2016) The development of malaria diagnostic techniques: a review of the approaches with focus on dielectrophoretic and magnetophoretic methods. Malar J 15:358. https://doi.org/10.1186/s12936-016-1400-9

12. Santana-Morales MA, Afonso-Lehmann RN, Quispe MA, Reyes F, Berzosa P, Benito A, Valladares B, Martinez-Carretero E (2012) Microscopy and molecular biology for the diagnosis and evaluation of malaria in a hospital in a rural area of Ethiopia. Malar J 11:199. https://doi.org/10.1186/1475-2875-11-199

13. Wongsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH (2007) A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). Am J Trop Med Hyg 77:119–127

14. Performance MRDT (2008) Results of WHO product testing of malaria RDTs: Round 1. World Health Organization, Geneva

15. Pinheirob VE, Thaithongc S, Browna KN (1993) High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 61:315–320. https://doi.org/10.1016/0166-6851(93)90077-b

16. Kain KC, Harrington MA, Tennyson S, Keystone JS (1998) Imported malaria: prospective analysis of problems in diagnosis and management. Clin Infect Dis 27:142–149. https://doi.org/10.1086/514616

17. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA (1999) A genus-and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg 60:687–692. https://doi.org/10.4269/ajtmh.1999.60.687

18. Snounou G, Pinheiro L, Gonçalves A, Fonseca L, Dias F, Brown KN, do Rosario VE (1993) The importance of sensitive detection of malaria parasites in the human and insect host in epidemiological studies, as shown by the analysis of field samples from Guinea Bissau. Trans R Soc Trop Med Hyg 87:649–653. https://doi.org/10.1016/0035-9203(93)90274-t

19. Krishna S, Bharti PK, Chandel HS, Ahmad A, Kumar R, Singh PP, Singh MP, Singh N (2015) Detection of mixed infections with Plasmodium spp. by PCR, India, 2014. Emerg Infect Dis 21:1853–1857. https://doi.org/10.3201/eid2110.150678

20. Makler MT, Palmer CJ, Ager AL (1998) A review of practical techniques for the diagnosis of malaria. Ann Trop Med Parasitol 92:419–433. https://doi.org/10.1080/00034899859401

21. Zakeri S, Kakar Q, Ghaseemi F, Raeesi A, Butt W, Safi N, Afshar Pad M, Memon MS, Gholizadeh S, Salehi M, Atta H, Zamani G, Djadid ND (2010) Detection of mixed Plasmodium falciparum & P. vivax infections by nested-PCR in Pakistan, Iran & Afghanistan. Indian J Med Res 132:31–35

22. Khattak AA, Venkatesan M, Khtoonyo L, Ouattara A, Kenefic LJ, Nadeem MF, Nighat F, Malik SA, Plowe CV (2013) Prevalence and patterns of antifolate and chloroquine drug resistance markers in Plasmodium vivax across Pakistan. Malar J 12:310. https://doi.org/10.1186/1475-2875-12-310

23. Tangpukdee N, Duangdee C, Wilairatana P, Krdosood S (2009) Malaria diagnosis: a brief review. Korean J Parasitol 47:93–102. https://doi.org/10.3347/kjp.2009.47.2.93

24. Hänscheid T, Grobusch MP (2002) How useful is PCR in the diagnosis of malaria? Trends Parasitol 18:395–398. https://doi.org/10.1016/s1471-4922(02)02348-6

25. World Health Organization (2016) Malaria microscopy quality assurance manual-version 2: World Health Organization

26. World Health Organization (2010) Basic malaria microscopy: tutor’s guide: World Health Organization

27. Zainabadi K, Adams M, Han ZY, Lwin HW, Han KT, Ouattara A, Thura S, Plowe CV, Nyunt MM (2017) A novel method for extracting nucleic acids from dried blood spots for ultrasensitive detection of low-density Plasmodium falciparum and Plasmodium vivax infections. Malar J 16:377. https://doi.org/10.1186/s12936-017-2025-3

28. MacKinnon A (2000) A spreadsheet for the calculation of comprehensive statistics for the assessment of diagnostic tests and inter-rater agreement. Comput Biol Med 30:127–134. https://doi.org/10.1016/s0010-4825(00)00006-8

29. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP (2003) Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Clin Chem Lab Med 41:68–73. https://doi.org/10.1159/000236737

30. Directorate of Malaria Control (2019) Pakistan malaria annual report 2019. Program management unit directorate of malaria control, Pakistan

31. Okoro C, Chukwuocha U, Nwakwo G, Ukaga C (2015) Presumptive diagnosis and treatment of malaria in febrile children in parts of south eastern Nigeria. Infect Dis Ther 3:240. https://doi.org/10.4172/2332-0877.1000240

32. Dhorda M, Ba EH, Kevin Baird J, Barnwell J, Bell D, Carter JY, Dondorp A, Ekwati L, Gatton M, Gonzalez I, Guerin PJ, Incarnato S, Litlye K, Manend D, Nosten F, Obare P, Ogutu B, Olliaro PL, Price RN, Pruek S, Ramsay AR, Reeder JC, Silamut K, Sokha C (2020) Towards harmonization of microscopy methods for malaria clinical research studies. Malar J 19:324. https://doi.org/10.1186/s12936-020-03352-z

33. Jaleta F, Garaona G, Gerfenes T (2020) Evaluation of malaria microscopy diagnosis performance in public hospitals of eastern and central part of Oromia Region, Ethiopia, 2019. Pathol Lab Med Int 12:1–8. https://doi.org/10.21203/rs.3.rs-21700/v1
34. Shahwani Z, Aleem A, Ahmed N, Mushtaq M, Afridi S (2016) A PCR method based on 18S rRNA gene for detection of malaria parasite in Balochistan. J Pak Med Assoc 66:1587–1591

35. Zaman S, Tan L, Chan HH, Aziz L, Abdul-Samat S, Wahid R, Kamal A, Ahmed M, Zaman V (2001) The detection of Plasmodium falciparum and P. vivax in DNA-extracted blood samples using polymerase chain reaction. Trans R Soc Trop Med Hyg 95:391–397. https://doi.org/10.1016/s0035-9203(01)90192-0

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.