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RESEARCH ARTICLE

Assessing the Efficacy of Simple and Molecular Diagnostic Methods in Tracking Malaria Parasite Proliferation (*P. vivax* and *P. falciparum*)

Muhammad Zahoor [a], Fazli Subhani [b], Murad Ali Khan [h]*, Majid Ayaz [d], Gul Laiba [c], Muhammad Junaid Ansari [e], Adnan Badshah [f], Shahzada Khurram Adrian Shah [g], Nauman Ahmad [i]

[a] *Department of Pediatric Research Institute, Children's Hospital of Chongqing Medical University, 136 Zhongshan, 2nd Rd, Chongqing 400014, China.*
[b] *Department of KP, Elementary and Secondary Education Peshawar, Pakistan.*
[c] *Department of Zoology Wildlife and Fisheries, MNS-University of Agriculture Multan, Pakistan.*
[d] *Department of Medical Laboratory Science and Biotechnology, Asia University, Taichung, Taiwan.*
[e] *Department of Pathobiology, Faculty of Veterinary Science, BZ University, Multan, Pakistan.*
[f] *Department of Parasitology, The University of Veterinary and Animal Sciences Swat (UVAS), Pakistan.*
[h] *Department of Parasitology, College of Veterinary Sciences, The University of Agriculture Peshawar, Pakistan.*
[g] *Department of Animal Health, The University of Agriculture Peshawar, Pakistan.*
[i] *Faculty of Veterinary and Animal Science, Gomal University, Dera Ismail Khan, Pakistan.*

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Corresponding author: **Murad Ali Khan**
Email: drmuradwazir@aup.edu..pk.

ABSTRACT

The current research to investigate the proliferation and comparison of rapid diagnostic tests, simple microscopic tests, and molecular diagnostic techniques for *P. vivax and P. falciparum* parasites. A total of 1,500 residents with suspected malaria patients through medical symptom and healthy people with no malaria symptoms were included in the study. The regular age of patients was 5-35 years, range from 5 to 35 years. The majority were 90 (60%) over 25 years. The study included 17 (78.0%) males and 30 (22.0%) females. The microscopy test results identified 331 cases for malaria infection, rapid diagnostic tests (Optimal method test) results identified 345 cases for malaria infection and *Plasmodium* infection has been confirmed in 299 cases. The 261 positive cases according to microscopic examination samples, but 38 positive cases were negative on the microscopic and SD-rapid test examinations were recoded respectively. It is concluded that the SD-rapid test examinations showed the best correlation with microscopy diagnosing of *P. falciparum* and *P. vivax* parasites. And the high compassion and specificity of Rt-PCR examination grasp great assure in malaria parasites identification and genus separation and should be implemented as a successful surveillance instrument for malaria monitoring, control and eradication in Bannu city.

1. Introduction

It is important to provide an accurate diagnosis of malaria sensible estimates of the shamble and prevention of malaria incorrect information interference [1, 2]. Many researchers, microscopic assessment of thick and thin blood stains failed measured a gold average for diagnosis of malaria, because its ability to classify infected *Plasmodium* spp and its low cost of parasitemia [1, 2, 3]. On the other hand, these techniques have many confines as it is time-consuming, labor-intensive and require the availability of highly trained

microscopes. Misdiagnosis parasitism can occur more commonly in cases of species misidentification, and this wrong treatment [4]. To conquer these confines, a number of alternative techniques for malaria diagnosing have been developed. One of them is the technique of parasitic DNA-based molecular detection [5,6]. Diagnostic based on polymerase chain reaction (PCR) the protocol is documented as a powerful detect tool and differentiate infections of mixed *Plasmodium* species high characteristic and sensitivity [7, 8]. Even so, owning one is still beyond the reach of the average person a number of confines, for example, high charge and low application in rural areas or field-based setting without proper laboratory apparatus [9]. Rapid Diagnostic Tests (RDTs) kit provides dependable consequences in a short time for parasite antigen detection and offers a helpful option to microscopy, in cases where PCR and microscopic assessment is not possible [10,11]. Because of these benefits, RDTs are introducing as a investigative tool in several malaria-edema regions. Conversely, many significant problem remain attention should be paid to the invasive field conditions, as well as diagnostic accuracy, high cost and efficiency [12]. RDTs kit offerings may also be artificial by the detection of remaining parasitic antigens from previous illness, resulting in false-positive results, and the elimination or mutation within the parasitic antigen, consequential in false positives [9]. Plus, it's available right now RDTs do not allow separation and quantification of *Plasmodium* spp. except *P. falciparum, P.vivax* [9, 10, 13].

In addition, in 2018, the highest number of malaria cases were reported by WHO provincial headquarters for Africa (213 million or 93%), followed by the WHO provincial headquarters for Southeast Asia (3.4%) and third, the WHO provincial headquarters for the Eastern Mediterranean (2.1%) [14]. *Plasmodium Spp*. including *P. Knowlesi, P. malariae*, and *P. vivax* are accountable for initiate malaria in humans [15]. On the other hand, *P. falciparum* is the most common parasite in the African county, secretarial for 99.7% of malaria cases in 2018 [14]. *Plasmodium vivax* has 53% worldwide, 11% Afghanistan and 8% Pakistan and maximum numbers (47%) in India [14, 16]. According to the WHO, the occurrence of *P. vivax* malaria in Pakistan at present is 84% while 14.9% P. *falciparum* and 1.1% mixed cases (both infection of *P. falciparum* and *P. vivax*) [14, 17]. The tribal regions of Khyber Pakhtunkhwa have the highest malaria cases [17].

Despite recent advances in malaria eradication, vaccination and antimicrobial treatment, malaria infections are still difficult to control because they have no specific diagnostics techniques. For this complications of malaria can be avoided only with early judgment and successful treatment. Microscopy is considered the gold standard for diagnosing [2, 3]. This is a cheap, easy and economical way to detect parasites. However, this method is labor-intensive, dependent on the trained eye, and time-consuming. Its position is mostly dependable in identify parasitism less than 50 parasites per µl blood or miscellaneous illness [3,18]. The finding of small parasitemia is important, especially in showing donors for blood transfusions in local region where there is a hazard of transmission of the disease to asymptomatic carrier recipients [17].

PCR has been establishing as a molecular diagnostic procedure for malaria parasites. Since the introduction of PCR-based procedures for the diagnosis of malaria, a number of methods have been urbanized. These methods are more susceptible to low parasitemia and joint parasitic infections of these techniques were developed to make malaria diagnoses more accurate as compared to RDTs Kit and microscopic examination [19, 20, 21]. Therefore, the article investigates the frequency of Kit, microscopy-based, and PCR diagnosis of malaria in adverse cases.

2. Materials and methods

2.1 Research district and health infrastructure

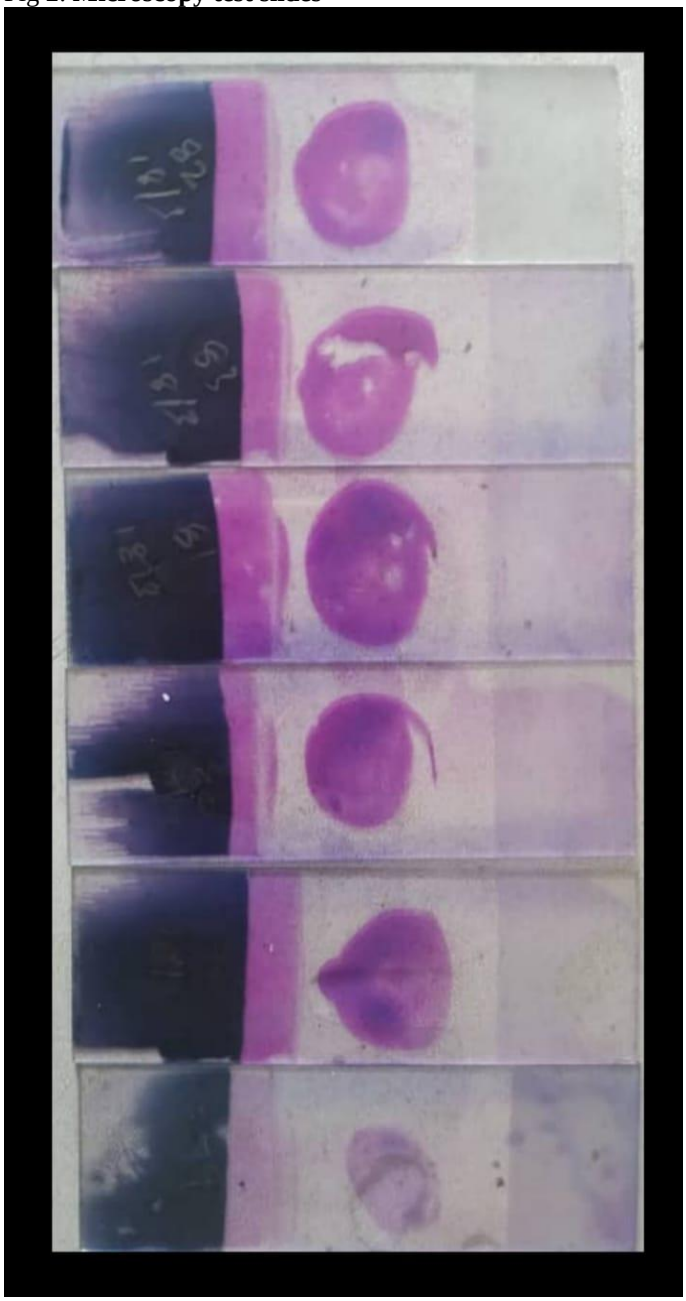
Between April 2019 and October 2019, field surveys were conducted for malaria in towns and villages in the Bannu city area. A total of 1,500 residents with suspected malaria patients with medical symptoms and 800 healthy people with no malaria symptoms were included in the study. Bannu regions of Khyber Pakhtunkhwa in Pakistan have long been a deserted area with imbalanced distribution of financial resources, poor livelihood conditions, and limited access to vaccines as compared to advance city. Malaria-infected or at-risk populations have grown significantly in these districts, as have malaria parasites **(Fig 1)**.

2.2 Microscopy test examination

3 ml of venous blood from each patient was collect in ethylenediamine tetra acetic-acid anticoagulated (EDTA)

bottles. The thick and thin blood stains were organized, stained with Leishman's stain, and the malaria parasite was examined by light microscopy using 100% oil immersion. The quantity of parasites per 200 white blood cells in a positive thick smear was counted. The parasite mass was uttered as multiple parasites / µl. Blood smears (thick and thin) were measured negative, if no parasites were observes. After 10 min of searching or testing 100 × high power under the microscope [22,23] (Fig 2).

Fig 2. **Microscopy test slides**



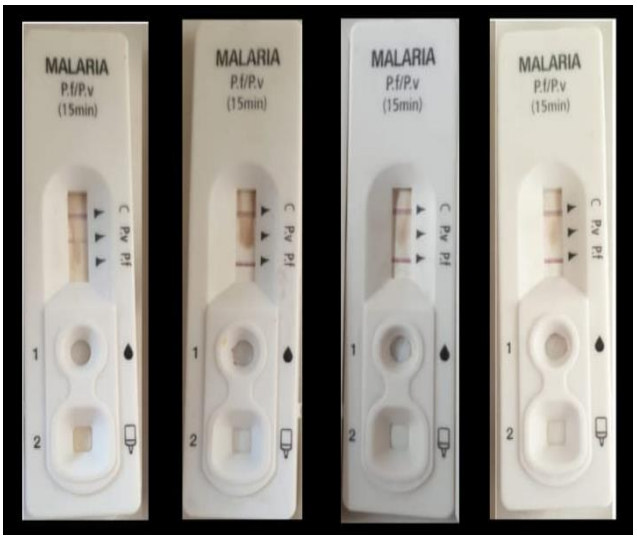
2.3 Rapid diagnostic tests (RDTs) examination

The similar blood samples were also experienced with the best measuring stick according to the preparation instructions [24]. Immunochromatographic assay malaria antigen kit (HRP2 (Pf) and pan pLDH (Pv) based) (SD Bioline Fob-Standard Diagnostics, Inc) detect the presence of pLDH antigen in lysed total blood. The pLDH is secret from live malaria parasites and the separation of *Plasmodium* spp is based on antigen variation among its isoforms. In addition to a controlled antibody the upper part of the test band has a reaction zone; the best dipstick has three test appearance or reaction zones. The 1st line exposed to the sample contains an antibody specific to *P. falciparum* pLDH. The 2nd and 3rd test line contains a pan-specific pLDH monoclonal antibody that identifies *Plasmodium spp* (*P. malaraie*, *P. vivax*, and *P. ovale*). Two drops of regent A (30µl buffer solution) were added to the conjugate well and four drops of reagent B clearing solution (8µl) were added. 10µl of blood were added to the conjugate well and gently varied. The measuring stick was placed perpendicularly in a conjugated well and acceptable to stand for 10 min. After 10 or 15min the bands were clearly visible. The consequences were explained directly (Fig 3).

Fig 3: 1. Positive - *P. falciparum and P. vivax*; one control band and 2 examination bands.

2. Negative - a control band at the top of the strip

3. .Positive - *Plasmodium spp* and *P. vivax*; A control band and two test band (mixed).



2.4 Polymerase chain reaction (PCR) examination

Samples were collected from inside and outside from government hospitals in EDTA cylinder, a blood (3ml) was collect from each patient. The DNA extracted from blood sample, using a DNA-purification Kit (Generator USA). Samples were collected as per the company protocols. The DNA extract was amplified between 36 samples with positive and negative controls

concurrently. Following the protocol proposed by the Real Time PCR according to Lee et al. [25], the investigation was carried out using the Taqman investigation. The primers used were specific to the genus; General Bank's affiliate number X 13926 for *P. vivax* and M 19172 for *P. falciparum* was used. The PCR 25 was performed through a pie-reaction mixture that included a DNA sample, a DNA test probe, a PCR mix, and a primer mix. Real-time PCR amplification was performed by thermal cyclist Rotor-Gene Q-series software 1.7 (QIAGEN company). Denatureation was originally done at 95 C° for 5 min. 40 cycles of denatureation were performed at 95 C° for 15 sec and annealing at 60 C° for 60 sec. Positive controls were prepared on microscopy by collecting +ve samples for *P. vivax* and *P. falciparum*. For the negative control, samples of healthy, aphrodisiacs that were –ve on the microscope were used. To analyze the compassion, the cyclic limit (c) of fluorescence was decided by sequential diversions. The consequence was explained by the examination of PCR quantization curves [25].

2.5 Clinical diagnosis

Following a detailed medical examination of patients, a standard case report form was developed to collect complete medical data on each patient's situation. In the case of children under five, the patient's history was collected from their family member.

2.6 Statistic analysis

The 95% confidence interval (CI) was considered reasonable and the value of ($P < 0.05$) was measured statistically significant.

3. Results

The standard age of patients was ranging from 5 to 35 years. The majority were 90 (60%) over 25 years. The study included 17 (78.0%) males and 30 (22.0%) females (Table 1). A whole of 1,500 patients, together with 717 suspected malaria patients, were tested for thick and thin blood simmer, using the presence of malaria parasites. Microscopic, microscopy test results identified 331 cases for malaria infection (Table 2). A whole of 1,500 patients, together with 717 suspected malaria patients, were tested for optimal blood method, using the presence of malaria parasites. Optimal method test results identified 345 cases for malaria infection (Table 3).

Table 1: Layout of the research contributors.

Individuality	Contributors (n=1500)
Age (years)	29.51 ± 8.73 (5-35)
Age Groups	
≤25 years	600 (40%)
>25 years	900 (60%)
Gender	
Male	17 (78%)
Female	30(22%)
History of fever within 24 hours (%)	100

Table 2. Microscopy test consequence

	Microscopic test					
	P.f	**P.v**	**P.f/P.v**	**Positive**	**Negative**	**Total**
Suspected patients	180	100	51	331	386	717
Healthy persons	00	00	00	000	783	783
Total	180	100	51	783	386	1500

P.f, *Plasmodium falciparum*; P.v, *Plasmodium vivax*

Table 3. Rapid diagnostic tests (RDTs) examination

	SD Rapid diagnostic tests					
	P.f	**P.v**	**P.f/P.v**	**Positive**	**Negative**	**Total**
Suspected patients	215	74	56	345	372	717
Healthy persons	000	00	00	000	783	783
Total	215	74	56	1128	372	1500

P.f, *Plasmodium falciparum*; P.v, *Plasmodium vivax*.

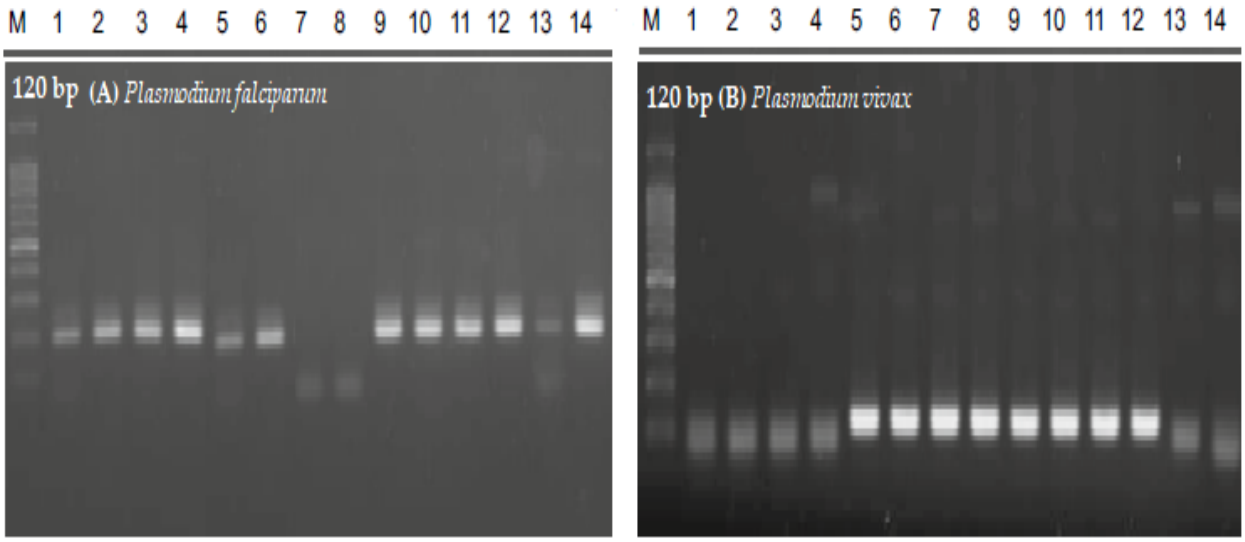


Fig 4. Identification of Plasmodium species by Rt- PCR analysis 1,500 blood samples were analyzed for *Plasmodium* DNA by Rt-PCR for *P. falciparum* and *P. vivax* (120 bp amplicons).

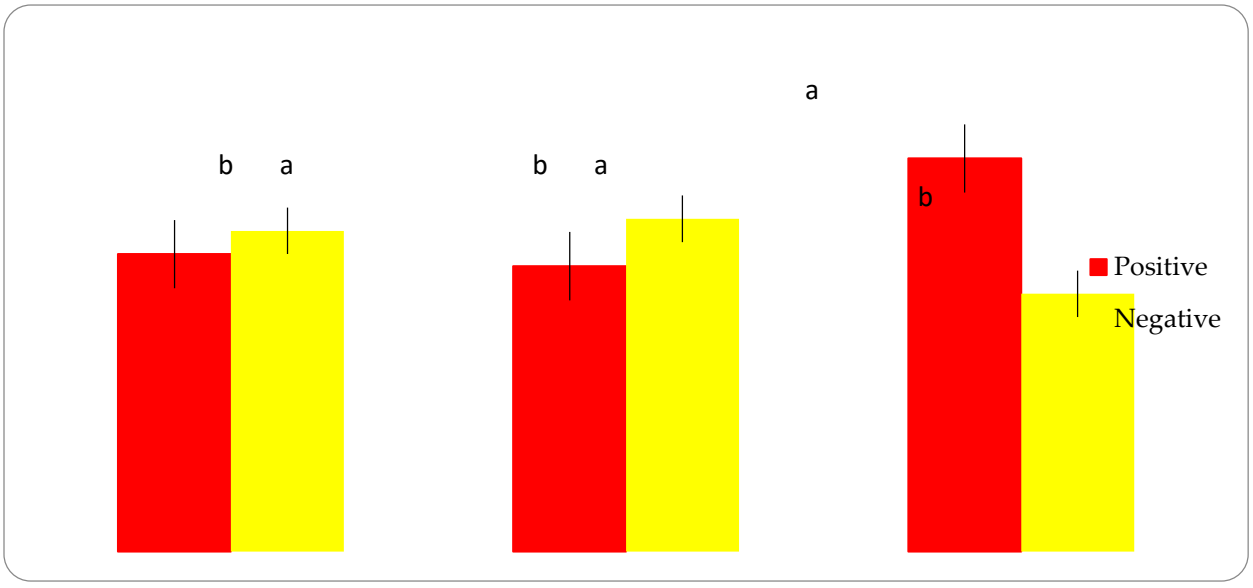


Fig 5. Comparison of the diagnostic presentation of SD rapid diagnostic tests, microscopic examination, and real-time PC

Table 5. Prevention of household characteristics to control malaria parasites.

A total of 1,500 blood samples were analyzed for *Plasmodium* DNA by Rt-PCR. Rt-PCR amplification specific to representative species the results of the

Plasmodium species are shown in Figure 4. Proper breeding for continuity Nucleotide sequence examination of randomly preferred PCR products from each *Plasmodium* spp. was established. The *Plasmodium* infection has been confirmed in 299 cases. The 261 positive cases according to microscopic examination samples, but 38 +ve cases were -ve on the microscopic and SD Rapid Test examination (Fig 4).

The majority of patients (75.3%) had un-diagnosed fever followed by recurrent fever (7.3%) and vomiting (6.0%). Only a small amount of patients had hemolytic anemia (4.0%), jaundice (1.3%), severe renal failure (1.3%) splenomegaly (4.0%), and 1 patient had hematuria (0.8%) (Table 4).

Table 4: Frequency of different patient signs and symptoms.

Patient signs and symptoms	Frequency	(%)
Acute Renal	25	1.3
Hemolytic Anemia	115	4.0
Jaundice	50	1.3
Recurrent Fever	345	7.3
Splenomegaly	100	4.0
Undiagnosed Fever Failure	650	75.3
Vomiting	200	6.0
Hematuria	15	0.8
Total	1500	100

Patients affected by both Plasmodium species generally have less formal education and less possession of wealth indicators (such as TV, facebook, Radio etc) (Table 5). About 75% people used 12V electric fan, 65% were using bed nets, 31% using repellent coils, and 20% reported using repellent fluids to control malaria parasites. Most importantly, in 47% of cases reported water stagnation in or near homes.

Table 5. Prevention of household characteristics to control malaria parasites.

S. No	Household characteristics	Percentage (%)
1	People follow treatment	99
2	Electric fan	75
3	Bed nets	65
4	repellent fluids	20
5	repellent coils	31

4. Discussion

The truthful identification of *Plasmodium* spp. is important not only for start a successful treatment modality, but also for scheming successful malaria manage procedures in local areas where a wide variety of there are malaria parasite [26]. Plasmodium mis-identification spp can pose serious public health concerns as they can prolong parasite approval period and lead to reproduction and medicine resistance [27]. Traditional microscopic examination by an authorized microscope has been adopted as the most important technique for diagnosing and monitoring malaria in Bannu districts. More recently, RDTs have been established as successful screening apparatus in field-based malaria survey because they offer readily obtainable consequences, and thus facilitate faster treatment [25]. On the other hand, only limited information is accessible on the efficiency of widely used parasite detection methods in the Bannu districts. Especially for those present who infected in *P. falciparum*, *P. vivax* and Pf/Pv, mixed, Bannu districts. A truthful diagnosis and prompt treatment of infected *Plasmodium* spp, are essential for the control and abolition of malaria in the country [28]. Consequently, research are required to evaluate the excellence and effectiveness of these diagnostic techniques in order to establish the best diagnosis of malaria and the results of nationwide malaria monitoring [24]. In current 1500 blood samples were collected from the different peoples of malaria affected areas in Bannu districts, which were examined under RDTs, microscopic examination and analyzed using Rt-PCR. On the whole malaria frequency as concluded by RDTs, microscopic test relatively similar while Rt-PCR method was more sensitive, with a consensus of 87.29%, but significant discrepancies were also noted between RDTs, microscopic test and Rt-PCR examination. The function of RDTs and microscopic test as a gold standard for malaria analysis was difficulty due to low parasitemia levels (less than 10-20 to 20-30 parasites per μl of blood) and a repeated fault in species classification in mixed infections has been given [29]. Low diagnostic consistency of microscopy for specific and mixed illness has already been accounted in local areas of Bannu districts. The current conclusion of malaria was compared with a new high-speed examination, Optimal, which is compared to the identification of malaria by conventional microscopy and Rt-PCR analysis and it turned out that the results of the comparison between the three techniques. A total of 1,500 peoples, including 717 patients suspected by malaria, were tested, using the presence of malaria parasites. The best method test consequences identified 345 cases of malaria infection. The fact that blood films detected some malaria infections that were not identified by optimal tests may explain the information is that Optimal identify pLDH, which is bent only by living parasites. It is achievable that a number of malaria patients self-medicated when symptoms appeared during the spread of malaria and did not report it to the presence physician [30]. There are more than a few probable explanations for this contradictions in

blood film tests and test results obtained through optimal tests, as well as (i) insufficient finding of low parasite rank by optimal, (ii) the information that optimal only identify form live parasites that produce pLDH, (iii) parasite detection, and (iv) false-positive responses [31,33].

This optimal test *P. falciparum* provides a more accurate diagnosis by identifying parasites infected with malaria that will be fail to spot through conventional blood film showing. An additional clarification might be that the patient's blood samples controlled a small number of parasites below the detection level of the optimal. According to Palmer et al., at this time; there are two other commercially accessible rapid kits for the diagnosis of *P. falciparum* malaria, Para-Sight-F and ICT tests. Although these two examinations offer a conclusion of malaria in less than 15 minutes, there is a difference between the three tests and the optimal test tested in previous research [34]. The initial variation is that ICT and ParaSight-F investigation are based on the discovery of a parasitic histidine-rich protein 2, which is present in the blood at least 28 days after starting antimicrobial therapy [35]. Thus, parasitic clearance after drug therapy cannot be diagnosed in patients. Those who have cleared the parasite may be misdiagnosed as malaria positive and may be given extra medication. The 2nd variation is that ICT and ParaSight-F examinations identify only one of the four types of *Plasmodium* that affect human [24,34,35].

The sensitivity of the three examinations were not the same in current research, as the Rt-PCR showed more positive cases samples than the rapid diagnostic test and the microscopy test. There are a few available studies on Rt-PCR examination. Many researchers have used different types of PCR, plain, nested, and Rt-PCR. It is probable that whole malaria cases are established by both microscopy and RDTs examination [21]. A little studies have been done in the Pakistani residents to conclude the digit of cases overlook throughout normal work, except they are considered to be a case of malaria. Such cases present an analytical confronts for the doctor and lead to treatment breakdown due to mismanagement [24]. This leads to displeasure on the fraction of the patient and a financial burden on the civilization [36]. Even though *P. vivax*-specific malaria is common in the region of Bannu districts and has been reported to have good results, a small number of cases of cerebral malaria have been reported with *Vivax* specie surroundings [37]. Therefore, considering malaria as benign, the possibility of life-threatening diseases cannot be ruled out. These findings make it wise to diagnose malaria at an early stage to avoid the negative cost of the illness [36]. However, the in general positive rate was significantly lower in all microscopy-negative patients using the PCR technique. But considering the malaria-related diseases and deaths, it can be said that microscopy in patients with severe medical suspicion of malaria is negative. PCR should be measured to verify the diagnosis. The results of current research also show that symptomatic patients with splenomegaly are more likely to be infected with malaria [33]. According to Mushtaq et al. [24] the majority of patients (75.3%) had undiagnosed fever. Only a little quantity of patients had hemolytic anemia (4.0%), jaundice (1.3%), severe renal failure (1.3%), and splenomegaly (4.0%). The PCR was positive in 3 (2.0%) cases results are similar to an additional local study by Iqbal et al [38] which reported that 3% of patients with a negative malaria parasite were established to be positive on PCR. The relatively large sample size of 2333 patients also reported 3.3% additional cases by PCR technique [24]. The Korean population-based study reported a 2.47% higher frequency of patients [39]. In Africa in 2009 (2.63%), in Thailand in 2006 Coleman et al [20] (1.17%), in 2007 Rodulfo et al [40] (0.49%) in Venezuela also observed a similar frequency of PCR positivity in microscopic negative samples. But a few other positive PCR cases observe a higher frequency found a 10% variation among the two techniques. This conflict between studies may be the consequence of differences in individual skills, as microscopy is a simply operator-dependent method. Furthermore, investigators use a variety of PCR methods that may influence the results of the study [24,34,35].

According to Kang et al, the most established cases were *P. vivax* and *P. falciparum* illness cases (420,462) and the ratio of *P. falciparum* and *P. vivax* infection was 70% [13]. Reliable with earlier findings, *P. vivax* and *P. falciparum* accounted for the majority of PCR-confirmed cases in this research. However, the ratio of *P. falciparum and P. vivax* was not as high as formerly information and the amount of miscellaneous *P. falciparum* was higher than *P. vivax* infection (17.4%) was recognized [19]. It is unspecified to *P. vivax* is suitable for the dominant spp in the study area with a current deficiency of *falciparum* cases of malaria, asymptomatic and sub-microscopic infection by 2 *Plasmodium* spps were in addition a major anxiety [13, 19].

The occurrence of *P. malaria* and *P. ovale* was little, but single and miscellaneous infections, including *P. vivax*, or *P. falciparum* were recognized. The distribution of *P. malaria* and *P. oval* in Myanmar failed reported earlier [27, 14]. Specifically, 8 confirmed *P. ovale* cases (*P. vivax* and *P. malaria*) was reported in the area of Pyin Oo Leary [43]. These clarification, beside with the current research, recommend that *P. malaria* and *P. oval* are widespread to upper Myanmar, although their frequency is not high. The *P. malaria* and *P. ovale* infection can be simply remembered for diagnosis by microscopic inspection, mainly because of very low parasitemia of parasites. Also, in miscellaneous infections with additional *Plasmodium* spp, it is harder to isolation of parasites as of characteristics with a huge quantity of parasites of new class [42, 44].

Anopheles in the district are familiarize to wastewater overflowing with dirty water, on the other hand, Stephensie strain mostly in spotless water vessels, and a river fluviatilis [17,45]. Just as *P. vivax* is very common in mountainous districts, so all the present intentional districts

are roofed with hills, which builds this district suitable for parasitic reproduction [46]. In addition, the high yearly rainfall in the areas under study also provides reproduction grounds for female mosquitoes to lay their eggs. We are unable to detect any *P. ovale* and *P. malaria* contamination in our trials. This result also agrees with the fact that these two genuses had not to be outdone in this region [17, 47]. In addition, 13% of cases affecting equally genus were established with jaundice, as reported in previous information [17,46]. Mostly Pakistan peoples clinical and biochemical findings from *P. vivax* and *P. falciparum* malaria show that most cases show an increasing proportion of hyperpiraxia (basal body temperature> 40˚C). They were used the following effect drugs such as, primaquine and chloroquine to treat *P. vivax* and *P. falciparum* spp, pyrimethamine or sulfadoxine (on day 1), and artisonate (for 3 days) were used according to the period of the patient and doctors advised. Artesonate or quinine was administered to treat severe malaria [17].

5. Conclusions

It is concluded that the SD-rapid test examinations showed the best correlation with microscopy diagnosing of *P. vivax* and *P. falciparum* parasites. And the high sensitivity and specificity of Rt-PCR examination holds great guarantee in malaria diagnosis and species clarification and should be implemented as a successful surveillance apparatus for malaria monitoring, control and eradication in Bannu city. Increasing speculation in socio-economic improvement, physically powerful health communications and malaria education are key interventions to decrease malaria in Bannu.

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Conflicts of interest
There are no conflicts of interest.

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