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ORIGINAL ARTICLE

Comparative Analysis of Rapid Diagnostic Test (ICT) and Microscopy for the Diagnosis of Malaria

FARID ULLAH[1], YASMEEN BIBI[2], MUHAMMAD ARIF[3], FOUZIA NAWAB[4], AYESHA WAKIL[5], SYED LUQMAN SHUAIB[6]
[1]*Medical Laboratory Technologist, Pure Health, Abu Dhabi, United Arab Emirates.*
[2]*Professor, Department of Physiology, Women Medical and Dental College, Abbottabad, Pakistan.*
[3]*Assistant Professor of Hematology, Department of Pathology, Jinnah Medical College, Peshawar, Pakistan.*
[4]*Ph.D Scholar, Department of Biochemistry, Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan.*
[5]*M.Phil Scholar, Department of Hematology, Institute of Pathology and Diagnostic Management, Khyber Medical University, Peshawar, Pakistan.*
[6]*Assistant Professor, Department of Pathology, Khyber Medical College, Peshawar, Pakistan.*
**Corresponding author: Dr. Syed LuqmanShuaib, Email: syedluqmanshuaib@yahoo.com*

ABSTRACT

Introduction: Malaria is caused by four Plasmodium species (*vivax*, *falciparum*, Ovale, and Malariae). Malarial parasites can be diagnosed by examining blood smear under microscope, rapid diagnostic test (ICT), and by detecting nucleic acid of parasite using polymerase chain reaction. This study was conducted to find out the diagnostic accuracy of rapid diagnostic test (ICT) in association with microscopy as a gold standard for the detection of malarial parasites P.*vivax*, P. *falciparum* and the effect of parasitic load on both diagnostic methods - ICT and light microscopy.

Materials & Methods: The study design was cross sectional validation, done at a private clinical set up in Peshawar, Pakistan. Parasite count was performed to quantify parasitic load strictly according to the rules constituted by World Health organization (WHO).

Results: A total of 32 patients were identified to be infected with malarial parasites on light microscopy and 68 cases were found negative. Among the positives, P.*vivax* was detected in 28 cases and P. *falciparum* in 4 cases. The ICT results showed 29 of the cases positive for malarial parasites and 71 negative. Among the positives, P.*vivax* was detected in 25 cases, P. *falciparum* in 2 cases, while 2 cases showed mixed infection. Thus the ICT result for malaria showed 95% sensitivity and 100% specificity with 97% of diagnostic accuracy. The parasitic count was found to be ≤ 208 in those false negative cases on ICT method which showed the impact of low parasitic load on the diagnostic accuracy of ICT method.

Key words: Malaria, Plasmodium *falciparum*, Plasmodium *vivax*, ICT

INTRODUCTION

Malaria is a serious and occasionally precarious disease that is caused by a parasite that infects female Anopheles mosquito (1). Malarial parasites can be transferred from a mother to the unborn baby ahead of or at the time of birth of the baby ("congenital" malaria) (2). As per the up-to-date *World Malaria Report*, issued in 2017, there were 216 million cases of malaria in 2016, which were 211 million in 2015. The death toll from malaria assessed was 445 000 in 2016, a comparable death toll in the former year (446 000) (4). Currently, certified statistics for malaria in India, accessible at NVBDCP, show 0.7- 1.6 million established infections of malaria and 400-1,000 losses of lives per annum (5). Approximately 60% population of Pakistan live in malaria-endemic areas. In Pakistan, 500,000 malarial disease and 50,000 deaths due to malaria occur every single year despite of well-developed malaria control program, with approximately 37% of cases in Tribal Areas, followed by Balochistan and Khyber Pakhtunkhwa provinces (6).

Plasmodium can be diagnosed by investigating thick and thin blood film through the microscope (7). Rapid Diagnostic Tests (RDTs) were first commercially introduced during 1994. Around 200 factory-made strips by sixty enterprises are presently being used and the effectiveness of some these devices is checked by international organization (WHO, 2012). The RDTs field developed very swiftly and continuous progress has been made in its technicalities with increase in their capabilities for malaria diagnosis. (10) It is suggested that the entire number of RDTs should be checked out with microscopy to verify the outcomes and if positive, to measure percentage of RBCs that are infested (16). There are number of devices which identify antigens produced by plasmodium parasites. This kind of immunologic ("immunochromatographic") tests devices are often used in a dipstick or cassette form, and provide results in two to fifteen minutes (17).The accuracy of ICT devices may be different significantly through various terrestrial parts which makes it challenging to associate the outcomes from researches undertaken under low quality settings.(13) The use of this ICT devices may reduce the time taken to find out that a patient is positive or negative for malaria.

The absence of resources or laboratory equipment in some malaria-endemic countries, in order to measure white blood cells (WBCs) of patients, rather an assumed WBCs of 8000/µL of whole blood has been established by the World Health Organization to assist in counting of malaria parasites in malaria patients (8).WBCs are very important in malaria involvement studies and managing patient. Counting of malarial parasites produces clinically helpful findings in managing patient and to observe the efficacy of medicine (19). Present study was conducted to evaluate the diagnostic accuracy ICT in association with microscopy as a gold standard for the diagnosis of malarial parasites P.vivex, P. *falciparum* and the impact of parasitic load/µL of whole blood on both the diagnostic methods ICT and light microscopy in those malaria positive cases.

MATERIALS AND METHODS

This cross sectional validation study was undertaken at a private clinical set up in three months duration from October 2017 to December 2017 in Peshawar district of Khyber Pakhtunkhwa, Pakistan. The study was approved by ethical committee of Institute of Paramedical Sciences, Khyber Medical University, Peshawar. 100 subjects from both genders and different age groups having temperature greater than 37° C at the time of coming to the clinic, with shaking chills in some individuals. Additional nonspecific indications like body ache, head pain, lethargy and abdominal distress were involved in the study using non probability convenient sampling technique. Consent was acquired from the patients or guardians as required. Those infected individuals who were cured for malaria in the former 4 weeks or with any other identified reasons of temperature like pharyngitis, tonsillitis,sinusitis and urinary tract infection were excluded from the study.

A venous blood sample of 2ml was taken from patient and added into EDTA tubes for microscopy and ICT testing. The data was collected on a predesigned pro forma for all the required variables and then entered into the excel sheet for analysis. ICT tests were carried out for each individual exactly according to company's instructions. The device was allowed to equilibrate at ambient temperature prior to testing. The device cassette was removed from the pocket and put on a fresh and angle free place. Using the dropper provided with the device, the blood was drained up to the fill mark (about 10 micro liters) or jester was used for the same purpose. The blood discharged to the sample spot (S) on the test device. Three complete drops (about 120 micro liter) of buffer were put into the buffer well. The test cassette is pre-coated with

specific antibodies to histidine rich protein II antigens (Pf HRP-II) of *P. falciparum*and lactate dehydrogenase specific antibodies (Pv.LDH) of *P.vivax*. The device was then observed for the manifestation of colored lines on the result window within (but not more than) 20 minutes. Positive test result was showed by the appearance of 2 stained lines, 1 on the control (C) position and the other on the test (T) position. A negative test result was showed by the manifestation of only 1 stained line on the control (C) position and no line on the test position. An invalid test is showed by the non-manifestation of stained line on the control position with or without a stained line on the test.

Thick and thin blood films were made as per the standard practices. A small drop of blood sample was smeared on microscopic slide. The thin blood film was fixed in methanol besides thick and then both were allowed to air dry and stained with Giemsa stain for 30 minutes. After that, the slide was washed on tap water from the stain and allowed to air dry in an oblique state and observed below oil immersion lens for plasmodium detection by qualified Medical Laboratory technologists who were unaware to the result of the ICT test. In 40 of the total 100 cases Light Microscopy was performed first then followed by ICT, while for other 60 cases ICT was performed first then followed by Light Microscopy. As a minimum 200 high power fields were observed before a patient test was given as negative. Both Thick and Thin blood smears were observed for each subject.

All the rules constituted by WHO were strictly applied where applicable. Asexual forms were counted except sexual (gametocyte) forms, which were not counted but just reported. In mixed infections, all asexual forms of plasmodium were counted at a time and the presence of several species were reported. Parasitic load was noted as a ratio of organisms to WBC in thick films. *Plasmodium* parasites count was calculated in contrast to 200 WBCs at the same time by pressing the allotted key as parasites or white blood cells were seen on the thick film ,using a multiple type tally counter. Five hundred WBC were counted where ≤99 parasites were counted after counting in contrary to 200 white blood cells. Where microscopists performed the parasitic count in the thin blood smears as a result of heavy parasitaemia (greater or equal to 100 parasites in thick smear /HPF), and parasites counted were recalculated with 200 white blood cells. Parasitic Load (parasite/µL of whole blood) were then calculated as parasite densities,using assumed WBC count of 8000/µL of whole blood established by WHO to be used appropriately in services that do not have the equipment to find out an individual's complete CBC parameters. The parasitic load was computed as=Number of parasites counted x 8000 white cells per µL divided by No. of white cells counted. A compound microscope, a multiple tally counter to count malaria parasites and to count white blood cells. Giemsa-stained blood slides to be examined, immersion oil and a handheld calculator.

The data along with demographic information was recorded on pre designed pro forma for statistical analysis. The data was gathered, entered into the excel sheet and interpreted by SPSS version 16. Study variables were gender, age, microscopy results, immune chromatography results and parasitic load. Mean standard deviation was calculated for continuous data like parasitic load and age of patient. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) were calculated by taking microscopy as gold standard.

RESULTS

In this study, a total 100 individuals were enrolled in which 59 (59%) were males and 41 (41%) were females. Their mean age was 23.44 year while maximum age was 57 year and minimum age was 4 year. Light Microscopy result showed that malaria was positive in 32 (32%) subjects and was negative in 68 (68%) of the total 100 subjects. Among the positive P. *falciparum* species was detected in only 4 cases with gametocyte the most prevailing Stage, while P. *vivax* species was found to be present in rest of the 28 cases with schizont- the most prevailing stage. ICT test results showed that malaria was positive in 29 (29%) individuals and was negative in 71 (71%) of the total 100 subjects that were tested for malaria on "HaelgenPf/Pan" ICT test devices. Among the positive P.*vivax* was detected in 25 cases while P. *falciparum* was detected in only 2 cases with the appearance of only one test line at P.f position of test window on ICT device and in rest of 2 cases the appearance of both the test lines on ICT device were observed which is termed as mixed infection of malaria. The diagnostic accuracy of ICT method was analyzed as the specificity was 100% and sensitivity was 95.6% for ICT. The Positive Predictive Value was 100% while NPV was 95.7% and the diagnostic accuracy of ICT method was 97% while the diagnostic accuracy of microscopic method was 100% and is shown in the Table 3.1. To see the impact of number of parasites on both the diagnostic methods in those positive cases, the parasitic count was done in those 32 positive cases on light microscopy strictly according to the WHO specified procedure and analyzed as the maximum parasitic load was 8320/µL of whole blood, minimum parasitic load was 112/µL of whole blood with mean of 3359.25 and is shown in the table 3.4. As there were total 32 positive cases on light microscopy and total 29 positive cases on ICT method so the three cases that were negative on ICT but positive on light microscopy, when parasite count was carried out in those three cases the number of parasites were very low (≤ 208 parasites/µL of whole blood) as compared to those cases that were positive on both the diagnostic methods which indicates impact of low parasitemia level on the diagnostic accuracy of ICT method.

Table 1: Diagnostic accuracy of ICT Method

		Microscopic Results		Total
		Yes	No	
ICT Results	Yes	29 (29%) TP	0 (0%) FP	29 (29%)
	No	3 (3%) FN	68 (68%) TN	71 (71%)
Total		32 (32%)	68 (68%)	100 (100%)

Table 2: Specificity, Sensitivity, PPV and NPV of ICT Method

Specificity	Sensitivity	PPV	NPV	Diagnostic accuracy of ICT
100 %	95.6 %	100 %	95.7 %	97 %

Table 3: Plasmodium species diagnosed in examination by Light Microscopy

Form of Parasite	Frequency	Percent
Negative cases	68	68.0
Falciperum positive cases	4	4.0
P.vivex positive cases	28	28.0
Total	100	100.0

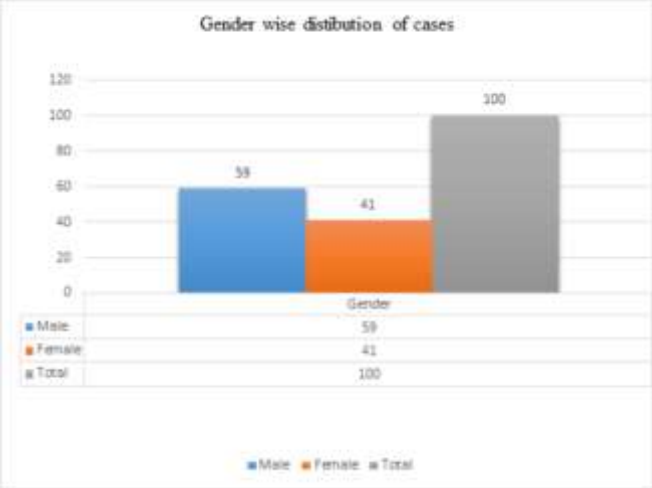
Table 4: Plasmodiumspecies diagnosed by ICT method

Form of Parasite	Frequency	Percent
Negative cases	71	71.0
Falciparum positive	2	2.0
Falciparum & P.vivex positive cases (Mixed Infections)	2	2.0
P.vivex positive cases	25	25.0
Total	100	100.0

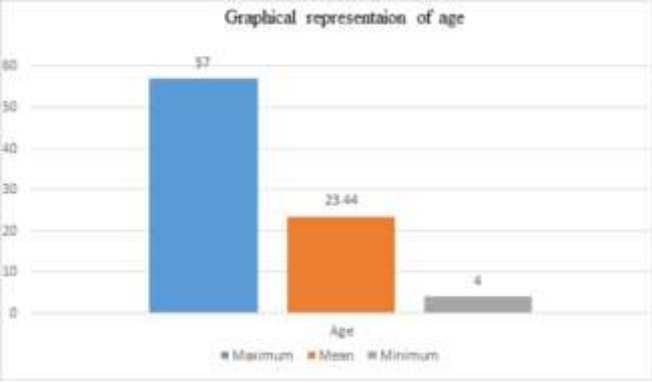
Table 5: Shows Parasitic load in whole Blood /200 or 500 WBCs

Number of parasites counted	Frequency	Percent
Negative	68	68.0
1056/µL	1	1.0
112/µL	1	1.0
1504/µL	1	1.0
1680/µL	1	1.0
176/µL	1	1.0
1840/µL	1	1.0
2016/µL	1	1.0
208/ µL	1	1.0
208/µL	1	1.0

2160/µL	1	1.0
2432/µL	1	1.0
3280/µL	1	1.0
400/µL	1	1.0
4320/µL	1	1.0
4520/µL	1	1.0
4800/µL	1	1.0
4840/µL	1	1.0
5080/µL	1	1.0
5680/µL	1	1.0
5840/µL	1	1.0
5920/µL	1	1.0
6320/µL	1	1.0
640/µL	1	1.0
720/µL	2	2.0
7360/µL	1	1.0
768/µL	1	1.0
7760/µL	1	1.0
7800/µL	1	1.0
816/µL	1	1.0
8200/µL	1	1.0
8320/µL	1	1.0
Total	100	100.0



Graph 1: Table/Graphical Representation of Gender



Graph 2: This figure shows age wise distribution of the subjects included in the study

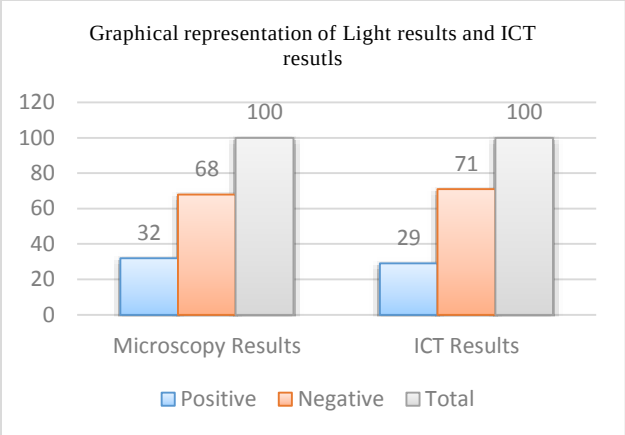


Figure 3: Comparison of results obtained with Microscopy and ICT method

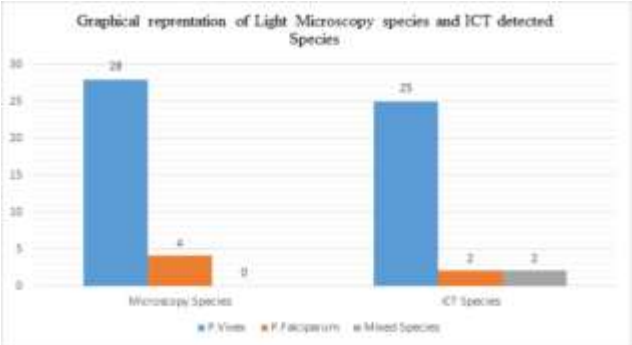


Figure 4: This figure shows species detected by Microscopy and ICT method

Table 6: Stages of Plasmodium parasites detected by Light microscopy

Forms of plasmodium	Frequency	Percent
Negative Cases	68	68.0
Gametocytes	2	2.0
Late rings	2	2.0
Ring &Gametocyte	1	1.0
Ring &Schizont	1	1.0
Rings	4	4.0
Schizont	16	16.0
Trophozyte	6	6.0
Total	100	100.0

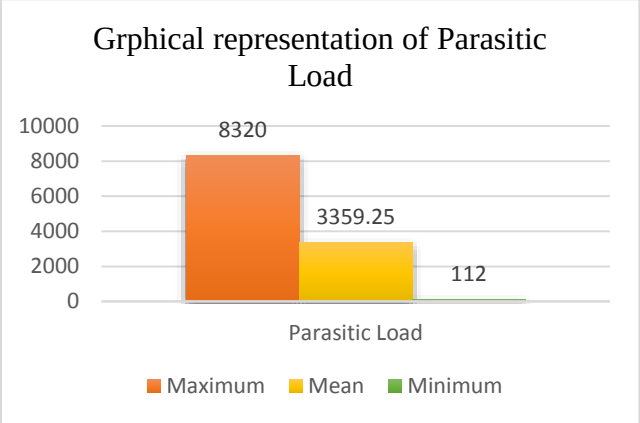


Figure 5:Graphical Representation of Parasitic Load

DISCUSSION

This cross sectional validation study was performed to compare ICT method with microscopic method for the detection of malarial parasite and to determine diagnostic accuracy (sensitivity and specificity) of ICT method in reference to microscopy as gold standard and also to see the impact of parasitic load on the diagnostic accuracy of both ICT and Light Microscopy. A total 0f 100 subjects were included in the study out of which 59 were male and 41 were female with maximum age 57 year, mean 23.44, and minimum 4 year. In my study the specificity was 100% and sensitivity was 95.6% for ICT method. The Positive Predictive Value was 100% while Negative Predictive Value was 95.7% and the diagnostic accuracy of ICT method was 97% while the diagnostic accuracy of Microscopic method was 100%.

A study conducted by Mohammad Nasar Khan et al, at Nowshera Teaching Hospital, Nowshera, Pakistan, with sample size of 161 patients (56% patients male, 44% patients female) showed that microscopic findings were analyzed as malaria was positive in 137 (85%) patients and was negative in 24 (15%) patients. ICT findings were analyzed as malaria was positive in 145(90%) patients and was negative in 16 (10%) patients. Diagnostic accuracy of ICT was analyzed as the sensitivity was 99%, specificity was 58%, PPV was 93%, NPV was 88% and the diagnostic accuracy was 93%. The study result showed that the accuracy of ICT was more in the diagnosis of malaria (20). In another study done at a private health center in district D.I.Khan, KPK, Pakistan by ZahidUllah et al,. In the course of the study period of eight months, a total of 470 patients were tested at the same time for microscopic examination and ICT for malaria parasite. Out of these 470 registered patients, 301 (64%) were males and 169 (36%) females. Among microscopy positive and ICT 184 (39.1%) cases were positive for P. *vivax,* 10 (2.1%) for P. *falciparum* and 276 (58.7%) cases were negative for both P. *vivax*and P. *falciparum*.The total sensitivity of ICT was 100 %, while specificity was 99.28%, with a PPV of 98.98% and NPV of 100%. Two cases of P. *vivax* revealed false positive results by ICT, perhaps due ingestion of anti-malarial drugs.In this study the performance of ICT was examined on malaria (Haelgen scientific LCC, USA) devices for the diagnosis of two prevailing species P. *falciparum*/P. *vivax* with microscopy. As a whole, ICT showed good sensitivity when associated with microscopy (21). In our study the sensitivity of ICT was 100%, specificity 95.5% and positive predictive value 100%, negative predictive value 95.7% while the overall diagnostic accuracy of ICT was 97%. In our study the diagnostic accuracy of microscopy is more than ICT method. Our study does not match with above studies, which is probably due the study setting, microscopic expert personals and techniques used in the studies. In another study the diagnostic accuracy of ICT was analyzed as the sensitivity was 97%, specificity was 60%, positive predictive value was 94%, negative predictive value was 75% and the diagnostic accuracy was 92%. The RDT had 97% sensitivity compared with 85% for the blood smear microscopy keeping PCR as the gold standard (22). In another study conducted at Uganda the sensitivity and specificity of an RDT was 75% and 90.6% respectively in the low transmission setting while in the high transmission setting the sensitivity reached to 93.5% and specificity dropped to 78.1%15 (23). In the study conducted at the China-Myanmar border area, the sensitivity of the RDT (pf/pan device) was 88.6% for plasmodium *falciparum* and 69.9% for plasmodium *vivax* (24). A study done by Hopkins *et al.*, in Uganda investigated that when all places were considered, the sensitivity of the HRP2-based test was 97% when associated with microscopy and 98% when rectified by PCR; the sensitivity of the pLDH-based test was 88% when associated with microscopy and 77% when rectified by PCR. The specificity of the HRP2-based test was 71% when matched with microscopy and 88% when rectified by PCR; the specificity of the pLDH-based test was 92% when matched with microscopy and >98% when rectified by PCR. Based on *Plasmodium falciparum* PCR-corrected microscopy, the positive predictive value (PPV) of the HRP2-based test was high (93%) at all but the place with the lowest transmission rate; the pLDH-based test and adept microscopy presented outstanding PPVs (98%) for all places. The NPV of the HRP2-based test was constantly high (>97%); in contrast, the NPV for the pLDH-based test fell significantly (from 98% to 66%) as transmission strength got high, and the NPV for skilled microscopy reduced significantly (99% to 54%) because of growing failure to detect subpatent parasitemia (25). There were two other studies in which RDTs also showed low sensitivity level (26, 27). The specificity, sensitivity and diagnostic accuracy of ICT method is good in above mentioned studies but in our study the diagnostic accuracy of ICT is less than that light microscopy but fair enough to be used in situations where expert microscopists are available. In another study conducted by Gabriella A et al., in Toronto, Ontario, Canada, the NOW® ICT assay test achieved increased specificity (> 95%) for each Plasmodium species and increased sensitivity for P. *falciparum* infections, but was less sensitive for the diagnosis of non-*falciparum* malaria species, especially at parasitemias < 1000 parasites/L. Further studies are necessary to establish the field performance of the test (28). The study undertaken by McMorrow et al., say that not withstanding increased sensitivity and specificity for *Plasmodium falciparum* infections, RDTs have numerous curbs that might decrease their value in low-transmission situations. They do not consistently identify decreased concentration parasitaemia (≤200 parasites/μL), several are less sensitive for Plasmodium *vivax* infections, and their capacity to identify *Plasmodium* ovale and Plasmodium malariae is unidentified. So, in eradication situations, different tools with greater sensitivity for low concentration infections (e.g. nucleic acid-based tests) are necessary to complement field diagnosis, and new highly sensitive and specific field-suitable tests must be established to warrant precise diagnosis of symptomatic and asymptomatic carriers. As malaria transmission drops, the percentage of low-density infections amongst symptomatic and asymptomatic individuals is likely to upsurge, which might confine the value of RDTs (29). The overhead studies have claimed that low level of parasitaemia decreases the diagnostic accuracy of ICT. In our study the parasitic load/μ of whole blood in those false negative was low (≤ 208/μL of whole blood) which is probably due to this low level of parasites in those cases.

As expert personals are not available especially in the peripheries of our country where malaria is endemic and it is tough to give accurate result about malaria, so it is suggested that the usage of ICT for detection of malarial parasites should be encouraged in hospitals and private medical laboratories, but it is a problem of ICT to accurately diagnose symptomatic and asymptomatic carriers with low level of parasitaemia. So additional research should be conducted to further enhance the diagnostic accuracy of ICT to accurately diagnose low level of prasitaemia. The sample size and duration of our study was small. The resources available were limited and only one type of device was used.

CONCLUSION

ICT Immune Chromatographic Technique in our study was not more accurate than microscopy in diagnosis of malarial parasites P.vivax and P. *falciparum* using only one company ICT devices. Even-though microscopy is a gold standard method for the diagnosis of malaria, yet it requires expert personals and techniques to retain 100% of diagnostic accuracy. Furthermore ICT is simple, economical and rapid as compared to light microscopy that needs expert microscopists to generate accurate results.

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