International Journal of Parasitology Research
ISSN: 0975-3702&E-ISSN: 0975-9182, Volume 8, Issue 1, 2016, pp.-168-172.
Available online at http://bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000241

THE USE OF MULTIPLEX PCR IN SPECIES IDENTIFICATION OF HUMAN MALARIA IN JAZAN AREA, SAUDI ARABIA

OMIMA M. EIDA*[1], AMANY M. EIDA[2] AND MOHAMMED M. EIDA[3]

[1]Departments of Parasitology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt
[2]Departments of Parasitology, Jazan University, KSA
[3]Departments of Tropical Medicine, Faculty of Medicine, Suez Canal University, Ismailia, Egypt
*Corresponding Author: Email-o_eida@yahoo.com

Received: December 13 2015; Revised: January 16, 2016; Accepted: January 17, 2016

Abstract- In Saudi Arabia, malaria is one of the major health concerns. *P. falciparum* and *P. vivax* is prevalent species in Jazan. Appropriate diagnosis and species identification is a very significant factor for convenient treatment of malaria. In general, microscopy is the standard method for diagnosis of malaria. However, polymerase chain reaction (PCR) assays display many possible benefits over microscopy as species identification of malaria especially in an era with few skilled microscopists. This study was conducted to compare microscopy and the multiplex PCR targeting the 18S rRNA gene for detection *Plasmodium vivax* and *Plasmodium falciparum*. A total 102 clinical blood specimens from suspicious malaria cases were gathered. Specimens were examined for the presence of *Plasmodium falciparum* and *Plasmodium vivax* by microscopy and multiplex PCR as well. The performance of multiplex PCR, OptiMAL test and microscopy was compared. The results revealed that 31.4%, 36.3% and 34.3% were positive by Microscopy, OptiMAL test and Multiplex PCR respectively. Multiplex PCR assay discovered two cases of *P. falciparum / P. vivax* mixed infections that cannot be detected by microscopic examination and OptiMAL test as mixed infections. Compared with microscopy, the sensitivity, specificity and test accuracy of OptiMAL test for detection *of P. falciparum* was 100%, 90.5% and 93.14% and multiplex PCR assay was 100%, 97.3% and 98.04% respectively. The sensitivity of OptiMAL test and multiplex PCR assay for detection of *P. vivax* was 50% and 75% respectively, while specificity of each method was 100%. Test accuracy of OptiMAL test and multiplex PCR assay was 98% and 99% respectively. In conclusions: our data showed that multiplex PCR is a sensitive, specific, and fast instrument so it can provide a beneficial differential diagnostic instrument for detecting *P. vivax* and *P. falciparum*.

Keywords- *P. falciparum; P. vivax;* Differential diagnosis; 18S rRNA; Multiplex PCR.

Citation: Eida Omima M., et al., (2016) The Use of Multiplex PCR in Species Identification of Human Malaria in Jazan Area, Saudi Arabia. International Journal of Parasitology Research, ISSN: 0975-3702 & E-ISSN: 0975-9182, Volume 8, Issue 1, pp.-168-172.

Copyright: Copyright©2016 Pervaiz A.P., et al., This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Introduction

Malaria is one of the extreme serious parasitic illnesses, particularly in tropical and subtropical areas. Malaria is a highly predominant disease in tropical and subtropical regions, affecting half of the world's population in 108 countries (216 million cases) with a death toll of more than 655,000 cases reported annually [1]. Four species of *Plasmodium* are the causative agents of human malaria; *P. falciparum* is responsible for most of the mortality, *P. vivax* causes significant morbidity and *P. malariae* and *P. ovale* are less prevalent [2]. In KSA, the disease is endemic to the southwestern part of the country, with the highest number of cases notified from Jazan and Asir regions. The highest percentage of cases reported among foreigners was in Jazan (25.32%) [3]. Simultaneously, there weren't any active cases of transmission of malaria parasite inside of the eastern region of Saudi Arabia [4]. In Saudi Arabia, *P. falciparum* and *P. vivax* are the commonest species [3] and malaria is considered one of the serious health problems. Therefore, a differential diagnosis of *P. falciparum* and *P. vivax* is very necessary for malaria control in KSA. Thus rapid and accurate diagnosis of infection and initiation of proper treatment are essential to improve outcome [4, 5]. It is critical to differentiate *Plasmodium spp*., since they differ greatly with respect clinical manifestations resulting from their unique biological features [6]. Examination of Giemsa-stained thick and thin blood films by conventional light microscopy is still considered the gold standard method [7]**.** Microscopy is a low cost diagnostic method; it is rapid and permits quantification and species identification [8,9]. However, blood film examination needs good expertise, especially at low parasitemia level. At present, it is well documented that microscopy is less sensitive than molecular techniques, and inaccuracy in species identification can happen with low parasitemia, mixed infection or modification by anti- malarial drug treatment [8, 10]. Immediate identification of *P. falciparum* and *P. vivax* is mandatory for proper therapy. An appearance of drug-resistant organisms to new therapy as artemisinin combination occur [11]. So, proper diagnosis is extremely important. Hypothetical therapy of malaria also leads to excessive use of antimalarial drugs. Therefore, a reliable assay that is capable of distinguishing different malaria parasites and to diagnose mixed infections would assist in efficacious treatment of the illness. Number of polymerase chain reaction (PCR) assays has been advanced and estimated [12,13]. These assays have shown a superior sensitivity compared to the conventional methods for detection of malaria parasite e.g. microscopy, immunochemical methods and dip-stick methods [13,14]. Although those PCR-based techniques are usually better in sensitivity and specificity, they are time-consuming and need sophisticated methods such as nested PCR or hybridization. It is, therefore, important to develop a convenient method for the diagnosis of malaria.

The present study aimed to evaluate multiplex PCR using primers targeting 18S rRNA genes for the differential diagnosis of *P. falciparum* and *P. vivax* isolates among patients from Jazan area, Saudi Arabia compared to microscopy as a gold standard.

Materials and Methods

Type of study:

The present study was conducted at the Parasitology laboratory, Jizan hospital, KSA while the molecular part of the study was performed at the Biotechnology Center, Faculty of Veterinary Medicine, Cairo University, Egypt.

Study Subjects:
The present study was performed from January 2014 to December 2014. A total of 102 patients aged 4–60 years, comprising 28 females (27.4%) and 74 males (72.5%), were enrolled in the study. Forty-five patients (44.1%) were Saudis while the remaining (57{55.9%}) were non- Saudis.

Microscopic examination: Before treatment, Blood samples were obtained from 102 clinically suspected malaria patients attending Jazan General Hospital in Jazan area, Saudi Arabia. All patients complained of fever with unknown origin associated in some cases with shivering, rigors and body aches for one day to 2 weeks. Blood sample (3 ml) was collected from each patient and examined for malaria by preparation of thin and thick blood smears for microscopic observation; the remaining whole blood was frozen at -20°C until use for PCR assay. Parasitemia per micro liter of blood was measured as parasite count = white blood cell count × parasites measured per 100 white blood cells)/100. The white blood cell count was 4,000. Samples of *P. falciparum* and *P. vivax* with high level of parasitemia were used as positive controls for both species for multiplex PCR. Negative control is a blank PCR mixture free of DNA. Thin and thick films were stained with 10% Giemsa and examined by two experienced microscopists examined the smears independently and were blinded to each other's interpretation.

Multiplex PCR:
Extraction of DNA of parasite was done from 0.1 ml of whole blood samples treated with EDTA using a QIAamp DNA blood kit (Qiagen Company). The extracted DNA was eluted in 0.2 ml of TE (10 mM Tris–HCl, pH 7.4 and 1 mM EDTA, pH 8.0) buffer and stored at -70^{0}C until use. The 18S rRNA gene was used for a differential diagnosis between *P. falciparum* and *P. vivax*. PCR amplification produced DNA fragments of the sizes of 1451-bp and 833-bp for *P. falciparum* and *P. vivax*, respectively. A forward universal primer (UF) was used (a genus-specific region), and reverse primers (VR for P. vivax and FR for *P. falciparum*) were used from a species specific region of *P. vivax* and *P. falciparum.* The primers of oligonucleotide have been developed before [15] and were as following: UF,5`-TCAGCTTTTGATGTTAGGGTATT-3`.VR, 5`-TAAACTCCGAAGAGAAAATTCT-3`. FR, 5`-GCATCAAAGATACAAATATAAGC- 3`. PCR was performed in a total volume of 20 µl consisting of a mixture of 2 µl of extracted DNA solution, 0.5 pmol UF, 20 mM dNTP each, 0.25 pmol reverse primers each (VR and FR), 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.0 mM $MgCl_2$, and 0.25 units of Taq polymerase (Sigma Aldrich Chemie GMBH, Deutshland). The reaction involved 35 cycles of 45 s of denaturation at 94 ^{0}C, 45 s of annealing at 57 ^{0}C, 45 s of extension at 72 ^{0}C and 5 min final extension at 72 ^{0}C. PCR product were analyzed on 1% agarose gel (Sigma Aldrich Chemie GMBH, Deutshland) and electrophoresed in 0.5 xT AE running buffer (0.5 mM EDTA, 20 mM Tris–acetate) containing 0.05% ethidium bromide for 30 min at 100 V in a Mupid-21 electrophoresis apparatus.

OptiMAL test:
The OptiMAL assay was performed according to manufacturer's instructions (DiaMed AG, Switzerland). In brief, 1 drop of whole blood was blended with 2 drops of lysis buffer A. Lysis buffer A damage red blood cells and discharges the plasmodium lactate dehydrogenase (pLDH). The samples were permitted to migrate to the highest point of the pLDH strip. The strip was set after 8 min in washing buffer B, which cleaned the hemoglobin from the strip. Interpretation of the assay results was performed promptly. Interpretation of the test results was as following: A monospecific antibody, which reacts only with P. falciparum, is found in reaction zone in the bottom of strip. When one control band and two test bands appeared at strip, the test was considered to be positive for *P. falciparum*. A second, pan-specific antibody, which identifies the pLDH of *P. vivax,* is just found above the level of this zone. When one control band and one test band appeared at middle of strip, the test was considered positive for *P. vivax.*
A third, a pan-specific monoclonal antibody is found at the highest point of the test strip and is used as a control for the test. When only one control band appeared at the top of the test strip without test band, the test was considered to be negative.

Statistical analysis:
Statistical analysis was performed with the SPSS version 16.

Specificity = $\frac{TN}{TN+FP}\times 100\%$,

Sensitivity = $\frac{TP}{TP+FN}\times 100\%$,

Negative predictive value = $\frac{TN}{TN+FN}\times 100\%$,

Positive predictive value = $\frac{TP}{TP+FP}\times 100\%$,

Test efficiency= $1\left(\frac{FP+FN}{total}\right)\times 100\%$ where TP = true positive, TN =true negative, FN = false-negative and FP =false positive results. Result of mixed infection with *P. falciparium* and *P. vivax* not included in Statistical analysis.

Ethical considerations:
All patients included in the study were informed by the aim of the study and consent was obtained from them. Proper treatment was prescribed for positive cases by the physician on duty

Results:
Microscopy:
Microscopy diagnosed 32/102 (31.4%) of the patients as malaria positive cases and 70/102 (68.6%) of the patients as negative. Out of the 32 infected cases, 28 (87.5%) were *P. falciparum*, 4 (12.5%) were *P. vivax* and there was no mixed infection (*P. falciparum* + *P. vivax*) [Table-2].

Multiplex PCR:
Multiplex PCR diagnosed 35/102 (34.3%) of the patients as malaria positive cases and 67/102 (65.7%) of the patients as negative. Out of the 35 infected cases, 30 (85.7%) were *P. falciparum*, 3 (8.6%) were *P. vivax* and two cases were mixed infection (*P. falciparum* + *P. vivax*) [Table-2]. Multiplex PCR and microscopy showed concordant results in 32 positive blood samples. Multiplex PCR detected 3 additional positive samples which were negative by microscopy.

OptiMAL test:
OptiMAL test diagnosed 37/102 (36.3%) of the patients as malaria positive cases and 65/102 (63.7%) of the patients as negative. Imported cases were 18/37 patients (48.6%). Out of the 37 infected cases, 35(94.6%) were *P. falciparum*, 2 (5.4%) were *P. vivax*. there was no mixed infection (*P. falciparum* + *P. vivax*) [Table-2].

Table-1 *Sex and Nationality of the patients*

Jazan	Sex		Nationality		Total No. (%)
	Male No.(%)	Female No. (%)	Saudi No. (%)	Non Saudi No. (%)	
Positive malaria cases	28(75.7)	9 (24.3)	19 (51.4)	18 (48.6)	37/102 (36.3)

Table-2 *Results of microscopy, OptiMAL test and a multiplex PCR in detection of P. falciparum and P. vivax.*

Species	Microscopy	Optimal test				Multiplex PCR			
		P.f	P.v	Mix^a	Total	P.f	P.v	Mix^a	Total
P.f	28	35	0	0	35	30	0	0	30
P.v	4	0	2	0	2	0	3	0	3
Mix^a	0	0	0	0	0	0	0	2	2
Total	32	35	2	0	37	30	3	2	35

Mix^a: mixed infection with *P. falciparium and P. vivax*

Table-3 *Diagnostic performance of OptiMAL test and multiplex PCR for detection of P. falciparum in relation to microscopy.*

Method*	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Overall Accuracy (%)
OptiMAL test	100	90.5	80	100	93.14
Multiplex PCR	100	97.3	93	100	98.04

*mixed infection not included

Table-4 *Diagnostic performance of OptiMAL test and multiplex PCR for detection of P. vivax in relation to microscopy.*

Method*	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Overall Accuracy (%)
OptiMAL test	50	100	100	98	98
Multiplex PCR	75	100	100	98.99	99

*mixed infection not included

Discussion:

Malaria is one of the major health concerns in Saudi Arabia. As a noteworthy wellbeing issue in the developing nations, malaria demonstrated to have a negative effect on the financial advancement. Different PCR assays have been used and assessed as an alternative procedure for diagnosis of malaria brought by different *Plasmodium* species [12,16]. Nested PCR tests diagnose asymptomatic malaria with minimum value of parasitemia [17]. On the other hand, nested PCR is moderately drawn-out and sophisticated [18]. The PCR-based microtiter plate hybridization (MPH) test was also introduced as presented as an alternative technique for diagnosis of malaria [19, 20]. The sensitivity of the test decreases at low parasitemia levels and the performance of this test required sophisticated processes. Recently, a multiplex PCR assay was utilized for identification different species of several parasites [21]. The point of preference of multiplex PCR in contrast with nested PCR is the amplification of DNA in a solitary tube (*P. falciparum* and *P. vivax*), in this manner avoiding contamination and reagents utilization is achieved [22].

The present study showed that 31.4%, 36.3% and 34.3% were malaria positive by Microscopy, optimal test and Multiplex PCR respectively. The species identification of positive cases was done by multiplex PCR and demonstrated that the more noteworthy number of malaria cases were *P. falciparum* (85.7%) while 3 (8.6%) were *P. vivax* and two cases (5.7%) were mixed infection (*P. falciparum + P. vivax*) which concurs with several researches [13, 23, 24]. Among them, 48.6% of total cases have been detected among foreigners. Many researches showed that many malaria endemic areas record mixed infections of these species. Also, the prevalence of mixed infections differs according the geographic area [25, 26]. Imported malaria is every now and again reported in KSA; most cases are *P. falciparum* infections. So, identification of *P. falciprum* and *P. vivax* is very vital for control of malaria in KSA [27]. Malaria cases among new comers, from either endemic regions or foreigners, were detected to be 97.45% of total cases. The highest percentage of cases among foreigners registered by Saudi Arabia Ministry of Health among foreigners was in the eastern region (25.91%) and Jazan (25.32%) [3].

In addition, the present study revealed that multiplex PCR test had the capacity to identify mixed infections that were not diagnosed by microscopy. This could be because of one species prevalence over another or that premature stage of *Plasmodium* species only present in blood of patient at the time of diagnosis by microscopy [28,29]. The multiplex PCR's sensitivity had the capacity to distinguish mixed infection while the microscopy test neglected to identify it. Hence, no doubt the multiplex PCR is a superior technique to distinguish mixed infections of *Plasmodium* species. The multipex PCR is the fundamental method that permits synchronous distinguishing proof and differentiation of human *Plasmodium* species. The reliability of the test was also being seen in other study as mixed infections were successful identified and further affirmed by sequencing data. The significant finding was that the real-time PCR did not appear to be as specific as the multipex PCR particularly in the diagnosis of mixed infections. A multiplex PCR is a simple and time-saving method [20].

Compared with microscopy, the sensitivity, specificity and test accuracy of multiplex PCR assay for detection *of P. falciparum* was 100%, 97.3% and 98.04% respectively. The sensitivity, specificity of multiplex PCR assay for detection of *P. vivax* was 75%, 100%. Test accuracy of multiplex PCR assay was 99%. This was similar to a study done by Khoa et al. (2003) [15] who proved the same results for multiplex PCR. This is also in accordance with a study done by Veron et al. (2009) [31] who found that other type of PCR as nested PCR and duplex real-time PCR tests displayed 100% sensitivity and specificity. In the present study, a comparative study of the microscopic and PCR test demonstrated that the results acquired by a PCR were better than those got by microscopy, in that all microscopy-positive specimens were observed to be positive by a PCR. While, three samples negative by microscopy were positive by PCR. This is similar to results of study done by Kritsiriwuthinan and Ngrenngarmlert (2011) [32] who proved that semi-nested multiplex PCR was more sensitive in malaria detection than microscopy. In present study, one sample was diagnosed as *P. vivax* by microscopy but negative by multiplex PCR. This may result from failure to amplify a PCR product due to poor quality of DNA or presence of mutations in the parasite genome [33] and \or inappropriate DNA isolation due to presence of inhibitors in the sample [34]. Microscopy is insufficiently sensitive to be utilized alone to screen malaria, especially in low level of parasitaemia. Microscopy could not identify malaria parasites when the density is 20–50 parasites/µl [35]. In spite of the fact that, microscopic examination of blood film is still gold standard, several drawbacks, for instance, requiring expert parasitologist, technical equipment maintenance, error in microscopist preparing and lacking quality control are connected with this technique [36]. Albeit master microscopy could hypothetically distinguish 10 p/µL parasitemia, normal microscopy has turned out to be not able in identifying lower than 100 p/µL parasitemia [37]. In summary, the multiplex PCR for the differential diagnosis between *P. vivax* and *P. falciparum* infections is highly beneficial where malaria has newly reemerged and fully-trained microscopists are restricted in number. Moreover, the PCR diagnostic test can easily be utilized for mass screening and field studies through automation and a PCR machine framework outfitted with a compact power supply.

Compared with microscopy, the sensitivity, specificity and test accuracy of OptiMAL test for detection *of P. falciparum* was 100%, 90.5% and 93.14%. The sensitivity, specificity and of OptiMAL test for detection of *P. vivax* was 50%, 100%. While, Test accuracy of OptiMAL test was 98%. Different studies throughout the world notified sensitivities of OptiMAL assay from as low as 25% to as high as 100% [38, 39, 40]. Albeit, high temperature (up to 60ºC) and dampness (up to 70%) were found not to influence results of test [41], false negatives were additionally observed even up to parasite densities of 2500 parasites/µL [40, 42]. The effectiveness of OptiMAL assay was extraordinarily affected by the level of parasitemia in patient's blood. The sensitivity of the OptiMAL assay was 96% at parasitemias of >500/µl; on the other hand, the sensitivity declined to 44 at parasitemias of <500/µl in a study done by Iqbal *et al*., [30]. This can conceivably be hazardous, as to miss the detection of malaria parasite in a patient having febrile illness may imply that complications develop because suitable treatment was not given to patients on time. The assessment of a negative result in this circumstance will be clearly impacted by the clinical manifestation [43]. In this study OptiMAL assay was positive in 2 samples which were negative by multiplex PCR. However, false-positive responses may happen in people who have high RF levels as notified before [44]. In present study, the OptiMAL assay was more sensitive in diagnosis of malaria patients with *P. falciparum* than *P. vivax*. This is in agreement with previous report [45]. On the other hand, a few reports had showed that The OptiMAL assay was more sensitive in diagnosis malaria infections with *P. vivax* than *P. falciparum* [46, 47]. OptiMAL assay has additionally been utilized in parasite diagnosis by non-invasive technique like saliva, but with

lower sensitivities. However, its sensitivity shows that it ought to not yet be viewed as first-line diagnostic test [43]. Delays in correct diagnosis or misdiagnosis that can prompt inappropriate treatment have been implicated in deaths among malaria patients in developed countries [48]. So, it is vital that a fast, sensitive and reliable technique should be accessible to laboratories for the diagnosis of malaria. This would obviate over diagnosis and over-treatment. Furthermore, it inhibits the increase of drug resistant strains of malaria parasite.

In the conclusion, in spite of the fact that microscopy is considered the backbone for routine diagnosis of malaria in Saudi Arabia, rapid diagnostic tests are important in crisis. However, according to the data from present study, OptiMAL test could not be dependably utilized to diagnose the mixed infections and it would be prescribed to utilize parasite particular antibodies to the specific antigens, instead of the pan-specific antibodies. This presses the requirement for new molecular method, which when utilized alongside microscopy, resulting in detection limits up to 1 parasite/µL. In spite the fact that the utilization of PCR at the field level is doubtful, using Whatman cards and collected blood spots in the field for removal to a lab and subsequent analysis make it easy. The ability to diagnose mixed infections also makes PCR a very efficient screening device for epidemiological studies.

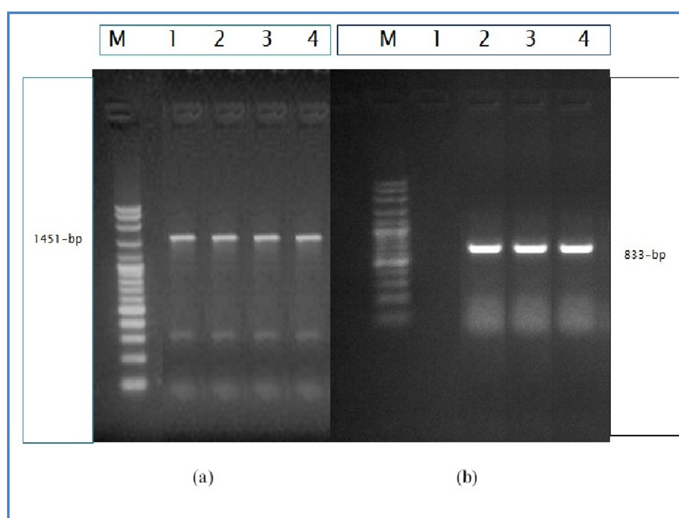

Fig-1 A multiplex PCR products from collected blood samples.
Fig-1a Lanes 1, 2, 3 and 4 represent *P. falciparum* (Band size 1451-bp);
Fig-1b Lanes *2, 3, and 4* represent *P. vivax* (Band size 833-bp)

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] World Health Organization (2010) World Malaria Report, 238.
[2] Goel A., Mangla A. & Singh T. (2014) *Pediatrics & Therapeutics,* 4, e 124.
[3] Ministry of Health. Health Statistical Year Book. Riyadh (KSA): Dar Al Hilal; 2010. Available from http://www.moh.gov.sa/Ministry/MediaCenter/News/Documents/healthybook.pdf
[4] Petti C.A., Polage C.R., Quinn T.C., Ronald A.R. & Sande M.A. (2006) *Clinical Infectious Diseases*, (42), 377-82.
[5] Bronzan R.N., McMorrow M.L. & Kachur S.P. (2008) *Molecular Diagnosis & Therapy,* (12), 299-306.
[6] Cnops L., Jacobs J. & Van E.M. (2011) *Clinical Microbiology* and *Infection*, (17), 1101–1107.
[7] Dajem S., Bin M., Ibrahim E. H., Mostafa O. M. S., Alshehri A., Kilany M., Aljeamelani A.A. & Heijan A. A. (2014) *Clinical Medicine and Diagnostics*, *4*(5), 99–105.
[8] Nandwani S., Mathur M. & Rawat S. (2005) *Indian Journal of Medical Microbiology,* (23), 176-8.
[9] Urgell A.R., Mueller D., Betuela I., Barnadas C., Iga J., Zimmerman P.A., del Portillo H.A., Siba P., Mueller I. & Felger I. (2010) *Malaria Journal.,* (9), 361 doi:10.1186/1475-2875-9-361.
[10] Hanscheid T. (2003) *Clinical Infectious Diseases*, 9:497–504.
[11] Dondorp A.M., Nosten F., Yi P., Das D., Phyo A.P, Tarning J., et al. (2009) *New England Journal of Medicine,* 361(5), 455-467.
[12] Mixson-Hayden T, Lucchi NW, Udhayakumar VP. (2010) BMC Research Notes 3, 88.
[13] El-Gayar E.K., El-Gayar A.K. & Nemr N.A. (2013) *Parasitologists United Journal,* 6 (1), 109-17.
[14] Wangai L.N., Karau M.G., Njiruh P.N., Sabah O., Kimani F.T., Magoma G. et al. (2011) *African* Journal *of Infectious Diseases,* 5(1), 1–6.
[15] Kho W.G., Yong Chunga J., Jeong Sima E., You Kimb M., Wook Kimc D., Jongwutiwesd S. & Tanabee K. (2003) *Parasitology International*, 52, 229–236.
[16] Farrugia C., Cabaret O., Botterel F., Bories C., Foulet F., Costa J.-M., & Bretagne S. (2011) *Journal of Clinical Microbiology*, *49*(6), 2191–5.
[17] Wang B., Han S.S., Cho C., Han J.H., Cheng Y., Lee S.K., Han E.T. *et al.,* (2014) *Journal of Clinical Microbiology*, *52*(6), 1838–45.
[18] Fuehrer H.-P., Fally M. a., Habler V. E., Starzengruber P., Swoboda P. & Noedl, H. (2011) *Journal of Clinical Microbiology*, *49*(4), 1628–1630.
[19] Chai J., Park Y., Guk S., Oh K., Oh M. & Lee S. (2000) *American Journal* of *Tropical Medicine* and *Hygiene,* 63(1,2), 80–84.
[20] Lee P., Chong E., Anderios F., AL Lim Y., Chew C. & Chua, K. (2015) *Malaria Journal*, *14*(1), 28. http://doi.org/10.1186/s12936-015-0542-5.
[21] Hu W., Wu S., Yu X., Abullahi A. Y., Song M., Tan L., *et al.,* (2015) *BioMed Research International*., ID 406168, 6 pages. http://dx.doi.org/10.1155/2015/406168
[22] Article O. (2015) *Iranian Journal* of *Parasitology,* 10 (1), 39–45.
[23] Dawoud H.A., Ageely H.M. & Heiba A.A. (2008) *Journal of the Egyptian Society of Parasitology,* 38(2):339-50.
[24] Azikiwe C.C.A., Ifezulike C.C., Siminialayi I.M., Amazu L.U., Enye J.C. & Nwakwunite O.E. (2012) *Asian Pacific Journal* of *Tropical* Biomedicine*,* 2, 307-10.
[25] Hamer D.H., Singh M.P., Wylie B.J., Yeboah-Antwi K., Tuchman J., Desai M., Udhayakumar V., Gupta P., Brooks M.I, Shukla M.M., et al (2009) *Malaria Journal*, 8, 210.
[26] Rasheed A., Saeed S. & Khan S.A. (2009) *Journal of the Pakistani Medical Association*, 59, 220-223.
[27] Bin Dajem S.M. & Al-Qahtani A. (2010) *Annals of Saudi Medicine,* 30, 187–192.
[28] Alves FP, Durlacher RR, Menezes MJ, Krieger H, Silva LHP & Camargo EP. (2002) *American Journal* of *Tropical Medicine* and *Hygiene,* 66(6), 641-648.
[29] Steenkeste N, Rogers W, Okell L, Jeanne I, Incardona S, Duval L, *et al.* (2010) *Malaria J*., 9(1), 108.
[30] Iqbal J, Khalid N&Hira PR. (2002) *Journal of Clinical Microbiology*, 40(12), 4675-4678.
[31] Veron V., Simon S. & Carme B. (2009) *Experimental Parasitology*, 121, 346–351
[32] Kritsiriwuthinan K. & Ngrenngarmlert W. (2011) *Journal of Vector Borne Diseases*, 48, 214–218.
[33] Rantala A.M., Taylor S.M., Trottman P.A., Luntamo M., Mbewe B., Maleta K., et al. (2010) *Malaria Journal*, 9- 269.
[34] Al-Soud W.A. and Radstrom P. (2001) *Journal of Clinical Microbiology*, 39,485–93.
[35] Bejon P., Andrews L., Hunt-Cooke A. *et al.* (2006) *Malaria J.,* 5, 104.
[36] Ohrt C., Purnom O., Sutamihardja M.A., Tang D. & Kain K.C. (2002) *Clinical Infectious Diseases*, 186, 540-6.
[37] Coleman R.E., Maneechai N., Rachapaew N., Kumpitak C., Soyseng V., Miller R.S. *et al.* (2002) *American Journal* of *Tropical Medicine* and *Hygiene,* 66, 379–83.
[38] Fryauff D., Purnomo, Sutamihardja M., Elyazar I., Susanti I., Krisin, *et al.* (2000) *American Journal* of *Tropical Medicine* and *Hygiene,* 63(3),139-145.
[39] Coleman R., Maneechai N., Ponlawat A., Kumpitak C., Rachapaew N., Miller R., *et al.*(2002) *American Journal* of *Tropical Medicine* and *Hygiene,*

67(6), 563-565.
[40] Playford E.G. & Walker J. (2002) *Journal of Clinical Microbiology*, 40(11), 4166-4171.
[41] Moody A. (2002) *Clinical Microbiology Reviews***,** 15(1), 66-78.
[42] Pakalapati D., Garg S., Middha S., Kochar, A., Subudhi A. K., Arunachalam B. *et al.,* (2013) *Asian Pacific Journal of Tropical Medicine*, *6*(5), 346–51.
[43] Gbotosho G.O., Happi C.T., Folarin O., Keyamo O., Sowunmi A. & Oduola A.M.J. (2010) *American Journal* of *Tropical Medicine* and *Hygiene,* 83(3), 496-501.
[44] Han L. A, Jang J.W., Cho C. H., Kim J. Y., Han E. T., Yun S. G. & Lima C. S. (2014) *Clinical Microbiology and Infection*, 52(10), 3784–3787.
[45] Iqbal J., Muneer A., Khalid N., & Ahmed M. A. (2003) *American Journal* of *Tropical Medicine* and *Hygiene, 68*(5), 624–628.
[46] Maltha J., Gillet P. & Jacobs J. (2013) *Clinical Microbiology and Infection,* 19, 399–407.
[47] World Health Organization. (2009) Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs: round 1. World Health Organization, Geneva, Switzerland.
[48] Stauffer WM, Cartwright CP, Olson DA *et al*. (2009) *Clinical Infectious Diseases*, 49, 908-13.