CHAPTER I

ESSAY

Worldwide estimates of the number of patients with acute febrile illness due to malaria have varied from 10 to 500 million annually. These estimates are imprecise, misleading, and low because of inadequate diagnosis and incomplete reporting. To approximate the number of febrile patients who might be parasitaemic in malarious areas is difficult and limited because of the lack of diagnosis capacity (BREMAN JG et al., 2001).

Described by BREMAN JG et al., 2001 that environmental conditions are one of the factors linked to the malaria toll e.g. tropical areas with warm temperatures, heavy rainfall, and high humidity, influxes of migrants and refugees. Human engineering projects, e.g., construction of dams can result in increasing mosquitoes breeding places.

Along the Thailand-Myanmar border area, malaria is one of the major public health problems. The magnitude of problem is increased due to topography, difficult communications from one place to another, population migration, existence of drug resistant *P. falciparum* malaria. Annual malaria cases recorded at the Thailand-Myanmar border ranged from 25,000 to 35,000 at out-patient departments and 7,000 to

11,000 at in-patient departments during 1992-1998. Average malaria deaths are approximately 400 cases annually (Thailand Country Report proposed in the Thailand-Myanmar Meeting on Collaborative Border Health Activities on Malaria / Tuberculosis / AIDS 2002).

In Thailand, total malaria cases in fiscal year 2001 with microscopically confirmed were 127,039. Out of these numbers, 67,749 or 53.3%were Thai cases and 59,290 or 46.7% were foreign national cases. Over 90% of malaria cases have been reported from 30 border provinces. The top three provinces are Tak (23,821), Mae Hong Son (5,094) and Kanchanaburi (4,376)(Malaria Division, Department of Communicable Disease Control, The Ministry of Public Health 2001).

Population mobility and malaria

Malaria is greatly affected by population movement pattern, both across international borders and across internal boundaries inside country. Regarding to the report of Mekong malaria by SEAMEO TROPMED 1999, the malaria cases in Thailand by Thai and foreign origin for the 3-year period 1996-98 according to the province where microscopic diagnosis was made. In some areas cases among foreign nationals exceed those among Thai. There has been an increasing proportion of malaria cases among foreign nationals in recent times.

Thai-Burmese border and drug-resistant malaria areas

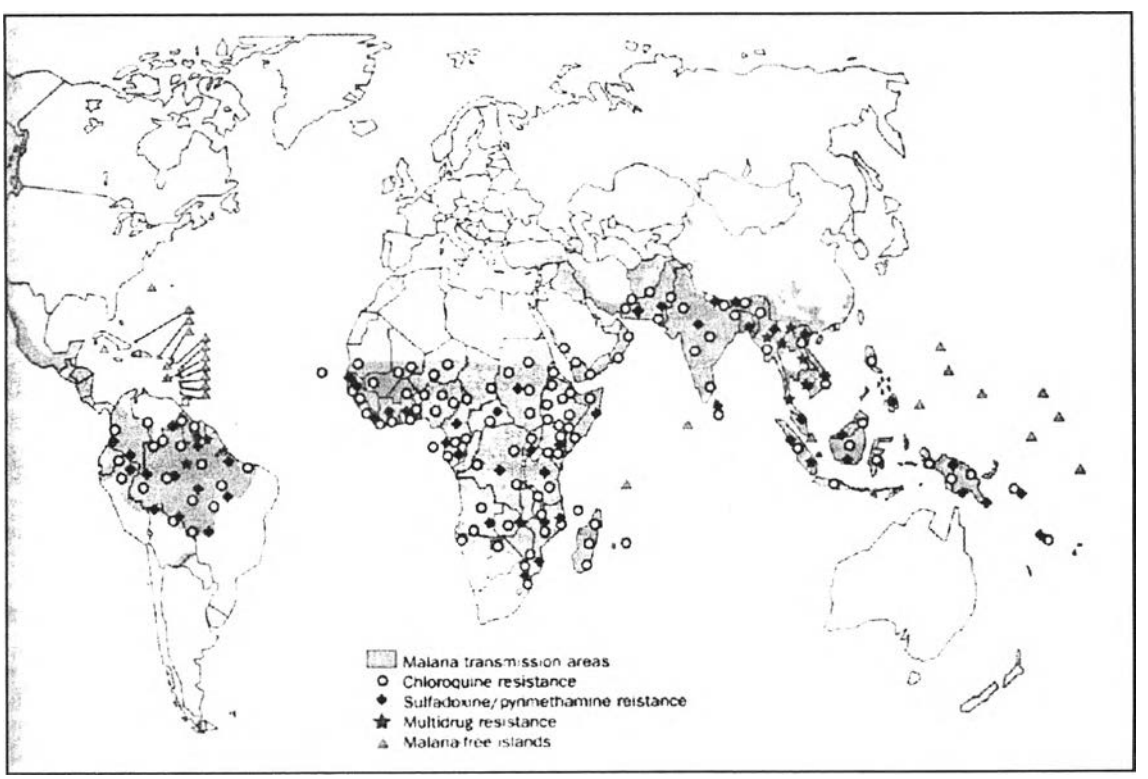
Drug resistance in malaria is an important public health concern and provides a negative impact on the control of malaria in affected countries especially in Thailand.

As mentioned in the review of epidemiology of drug-resistant malaria by C. WONGSRICHANALAI et al., 2002 that described on the development of malaria burden caused by drug-resistance which mostly occurred from Southeast Asia region especially along the Thai border. By the late 1980s, resistance to sulfodoxine-pyrimethamine and to mefloquine was prevalent on the Thai-Cambodian and Thai-Myanmar borders, rendering them established multi-drug resistant areas. Resistance of *Plasmodium falciparum* to chloroquine was first observed almost 50 years ago. In the late 1950s, resistance to chloroquine was noted on the Thai-Cambodian border and in Columbia. Today, chloroquine resistance occurs almost everywhere that *P. falciparum* does. In the mid-1960s, resistance to sulfadoxine-pyrimethamine was first noted on the Thai-Cambodian border and it became an operational problem in the same area within a few years of its introduction to the malaria-control program in 1975. For quinine, although recrudescence strongly suggestive of quinine resistance was noted in Brazil nearly 100 years ago,

Successive observations of clinical resistance to quinine only began to accumulate during the mid-1960s, especially from the Thai-Cambodian border. Mefloquine resistance was first observed near the Thai-Cambodian border in the late 1980s. The advent of mefloquine resistance in Thailand may have been influenced by the heavy use of the chemically related drug quinine just before the introduction of mefloquine. Mefloquine alone is no longer effective on the Thai-Myanmar and Thai-Cambodian borders, although it is still operationally useful in most other endemic areas in and around Thailand with field efficacy of more than 75%. Consequently, established multi-drug resistance occurs mainly in Southeast Asia, particularly the

border regions of Thailand. Since 1995, high-level mefloquine resistance led to replacement of this drug alone with the combination of artesunate and high-dose mefloquine for microscopically confirmed cases of falciparum malaria in established multi-drug-resistant areas of Thailand. These areas include Maesod on the western border with Myanmar and an area on the southeastern border with Cambodia. One study by F. NOSTEN et al., 2000 conducted in a confined population near Maesod suggested that use of artemisinin derivatives in combination with mefloquine might have reduced mefloquine resistance.

Figure 1 : Areas with reduced susceptibility of *P. falciparum* to chloroquine and sulfodoxine-pyrimethamine (SP) and areas designated as multi-drug resistant according to WHO. (From The Lancet Infectious Diseases Vol. 2, 2002)



As living in the areas described above, the Karen people in eastern Myanmar and western Thailand, multi-drug resistant falciparum malaria is the most important medical problem, despite relatively low levels of transmission (< 1 infection per person per year)(LUXEMBURGER et al., 1996)

To control the disease, rapid and precise diagnosis of malaria is important (M.T.MAKLER et al., 1998) To diagnose malaria, clinical diagnosis is unreliable because the malaria symptoms are very non-specific and overlap with those of other febrile illnesses. This statement supported by the study of LUXEMBURGER et al., 1998 that clinical features cannot predict a diagnosis of malaria or differentiates the infecting species in children living in an area of low transmission. The study of LUXEMBURGER et al., 1998 suggested that undiagnosed falciparum malaria in this area would increase morbidity and possibly mortality from malaria, and would also increase the spread of highly drug resistant strains of *P. falciparum*. Malaria should be confirmed by microscopically examination of a blood slide, as the results provide both malaria diagnosis and species differentiation.

In malaria patients, a prompt and accurate diagnosis is the key to effective disease management. The two diagnostic approaches currently used most often, however, do not allow a satisfactory diagnosis of malaria. Clinical diagnosis, the most widely used approach, is unreliable because the symptoms of malaria are very non-specific. Microscopic diagnosis, the established method for laboratory confirmation of malaria, presents technical and personnel requirements that often cannot be met, particularly in facilities at the periphery of the health care system. In addition, delay in

the provision of the microscopy results to the clinician mean that decision on treatment may be taken without the benefit of the results (WHO/USAID et al., 1999).

Confirmation of the diagnosis of malaria by microscopy gives an additional, critical advantage: identification of parasite species. Some rapid immuno-diagnostic (dipstick) methods will also achieve this. The importance of doing so is absolute, since severity of illness, mortality and correct drug therapy depend on parasite species recognition, whereas clinical diagnosis can only make a guess in deciding between malaria and other causes of fever, or in making appropriate therapeutic drug selection, without any comment on parasite species (P. SINGHASIVANON et.,al 1999).

To determine whether there is a need for my study, I will review all of the practical techniques using in malaria diagnosis based on M.T.MAKLER et al., 1998, MOODY A. et al., 2002, WONGSRICHANALAI C. et al., 2001 and WHO/MAL/2001.1091.

Light Microscopy: Thick and Thin Blood Smears

Developed in the 1900s and being considered as the gold standard of malaria diagnosis, microscopy offers many advantages.

- It is sensitive when used by skilled technicians : parasitaemia of 50 parasites/μL can be detected.
- It is informative. Parasites can be characterized in terms of species (*P.falciparum, P.vivax, P.ovale, and/or P.malariae*) and of stage of development (e.g. trophozoites, schizonts, gametocytes). In addition, the

parasite density can be quantified. Such quantification is needed to assess hyperparasitaemia (> 4 % of RBCs parasitised), which may be associated with severe malaria.

- Microscopy can be used to diagnose other disease such as tuberculosis, intestinal parasites, S.T.D, filariasis, urinary tract infection and etc.
- It is relatively inexpensive. Cost estimates for endemic countries range from about US$ 0.12 to US$ 0.40 per slide examined.

Therefore reliable microscopy is very difficult to implement in remote areas with difficulty of access. Moreover, microscopy also has many disadvantages.

- It requires well-trained and experienced technicians
- It is logistically demanding: a regular supply of reagent and good maintenance of material and microscope is needed.
- Proper and regular quality control (QC) must be organized.
- It is labor-intensive and time-consuming.

In addition, under-trained microscopists and lack of microscopes and staining material limit the usefulness of blood smear examination in many malaria endemic areas. Processing and reading large numbers of blood smears during malaria outbreaks increases error dramatically.

Fluorescent Microscopy

The quantitative buffy-coat (QBC) method (QBC®; Becton Dickinson, Franklin Lakes, NJ), the Kawamoto Acridine-Orange (AO), and the benzothiocarboxypurine

(BCP), these techniques are rapid and easy to perform when there are > 100 parasites/ μ L and provided sensitivities and specificities equivalent to examination of stained thick smears. An important limitation of methods based on AO and BCP is their inability to differentiate between *Plasmodium spp.*; in addition, AO is considered hazardous and has special disposal requirements, making it inappropriate for use in the field. The QBC method requires a particular centrifuge and centrifuge tubes and this increases cost to almost 2 US$ / sample.

In resource-poor areas, money to purchase even regular microscopes is often difficult and fluorescent microscopes are considerably more expensive.

Therefore the price of the fluorescent microscope could be a limited factor for laboratories interested with these methods especially those in the field.

Detecting Specific Nucleic-Acid Sequences

The detection of nucleic- acid sequences specific to *Plasmodium* parasites or PCR- based techniques has major advantage to detect infection with low parasitaemias e.g. at five parasites/μL. However, the usefulness of PCR technology is limited by the need for expensive, specialized, laboratory equipment, personnel trained in genetic technologies and 'clean room' facilities, and by the high cost of the enzymes and primers used. These factors prevent widespread use of PCR-based methods in developing countries, especially in the remote of malarious areas.

Antigen Detection

Because the antibody-detection tests for malaria were limited in sensitivity and the ability to distinguish active from prior infections, the antigen capture tests were introduced. These tests can be performed in about 15 minutes and the development of a colored test line indicates a positive result. There are two parasites antigen currently used in the rapid diagnostic tests;

- Histidine-rich protein 2 (HRP-2) e.g. ParaSight F (Becton Dickinson, USA), ICT *Pf* and ICT *Pf/Pv* (AMRAD, Australia), Paracheck Pf (Orchid Biomedical Systems, India). Since **only *P.falciparum* releases HRP-2**, these tests will give negative results with patients infected with *P.vivax, P.ovale*, or *P.malariae*.
- Parasite Lactate Dehydrogenase (pLDH) e.g. OptiMAL (Diamed, Switzerland). This test can detect *P.falciparum* and non *P.falciparum (P.vivax, P.malariae and P.ovale*) but cannot detect mixed infections or distinguish between *P.vivax, P.ovale* and *P.malariae*.

HRP-2- based Serological Assays

As described above, the limitation of HRP-2 targeting tests is only *P. falciparum* releases HRP-2 in the blood circulation so the test gives negative results for sample containing *P. vivax, P. ovale* or *P. malariae*. Many cases with non- falciparum malaria will be misdiagnosed as malaria negative.

Another limitation is HRP-2 persist in blood long after the clinical symptoms have disappeared and the parasites have been cleared from the host after a proper treatment.

pLDH-based Serological Assays

As described above that this test can detect *P. falciparum* and *non-falciparum* infection. Therefore, this test is very useful in areas where more than one species of *Plasmodium* is presenting. It can be used to identify the *Plasmodium species* infecting the patients in each village quickly and allow public health workers to deliver the most appropriate treatment according to *P. falciparum* or *non-falciparum* infection. Although, the test cannot distinguish mixed infection owing to the common capture obtained with the pan specific antibody band, its ability to identify *falciparum* infection provide a benefit to prevent patients from fatal infection.

Table 1: Selected recent field studies that evaluated the accuracy of malaria dipsticks

Dipsticks	Standard	Population	Maiaria prevalence(%)	Sensitivity (%)	Specificity (%)
Tests that detect *Plasmodium falciparum* only					
ParaSightF	GTS	The Gambia	61(85/139)	97	91
ParaSightF	GTTS	Tanzania	52(209/400)	98 100	95 94
ParaSightF and ICT Malaria Pf	GTS	Uganda	43(78/180)	≥ 100/μL ParaSightF 96 ICT 100	ParaSightF 96 ICT 92
ICT Malaria Pf	GTTS	Cameroon	50(100/199)	98	83
ICT Malaria Pf	GTTS	Kenya	16(26/164)	81	96
ICT Malaria Pf	GTTS	Thailand	17(97/551)	>300/μL 96 61-300/μL 67	96
PATH Test	GTS	Senegal	70(127/182)	96	93
Determine Malaria Pf	GTS	India	50(262/526)	98	87
Tests that detect both *P. falciparum* and non-*P. falciparum*					
BD for Pf and Pv	GTTS	Thailand and Peru	Pf 20(170/870) Pv27(234/870)	Pf 98 Pv 87	Pf 93 Pv 87
ICT Malaria Pf/Pv	GTTS	Indonesia	Pf 44(247/560) Pv 9 (49/560)	Pf 96 Pv 75 (96 if > 500/μL)	Pf 90 Pv 95
OptiMAL	GTS	Honduras	Pf 8 (17/202) Pv 49(99/202)	Pf 88 Pv 94	Pf 99 Pv 100
OptiMAL	GTTS	Thailand and Peru	Pf 17(313/1887)	Pf 91 Pv 83	Pf 95

(Reviewed by WONGSRICHANALAI C. from Research Update *TRENDS in Parasitology* VOL.17 No. 7 July 2001)

Lactate Dehydrogenase and the Diagnosis of Malaria (M.T.Makler, R.C. Piper and W.K.Milhous 1998)

Over the past years, several methods have been developed to make the differences between *Plasmodium lactate dehydrogenase* (pLDH) and the human LDH isoforms for the purposes of measuring pLDH in blood and in vitro cultures. These methods have been incorporated into an easy screening method for the identification and quantification of parasite growth in in vitro cultures using a Malstat reagent. In addition, a rapid immunochromatographic method, the OptiMAL assay Blood-stage malaria parasites synthesize lactate dehydrogenase (LDH) as the terminal enzyme in their glycolytic pathway. The *Plasmodium* LDH (pLDH) isoforms can be distinguished from the other isoforms on the basis of unique epitopes within the pLDH protein, as well as on its enzymatic characteristics. The OptiMAL assay detects pLDH as an antigen and differentiates the type of malaria infection based on the reactivity with the panspecific and falciparum-specific antibodies. While dipstick tests are non-quantitative, the OptiMAL assay also appears to follow closely the level of parasitaemia, becoming negative on the day a patient is found to be parasite free after antimalarial therapy. As with other rapid immunochromatographic tests, which capture and detect the histidine-rich protein-2 (HRP-2) antigen, the OptiMAL assay is sensitive to ~ 200 parasites per microliter of whole blood, and in numerous field studies has shown to be ~ 95% sensitive for this parasitaemia. In addition, the OptiMAL assay is

able to distinguish *P. falciparum* infections from non-falciparum infections; a valuable feature where malaria species infections are endemic.

Figure 2 : demonstrates the principle of the dry, immuno-capture, OptiMAL assay. This assay uses two monoclonal antibodied, one specific for *P. falciparum* and the other recognizing all four *Plasmodium spp.* infecting man.

The OptiMAL detection assay

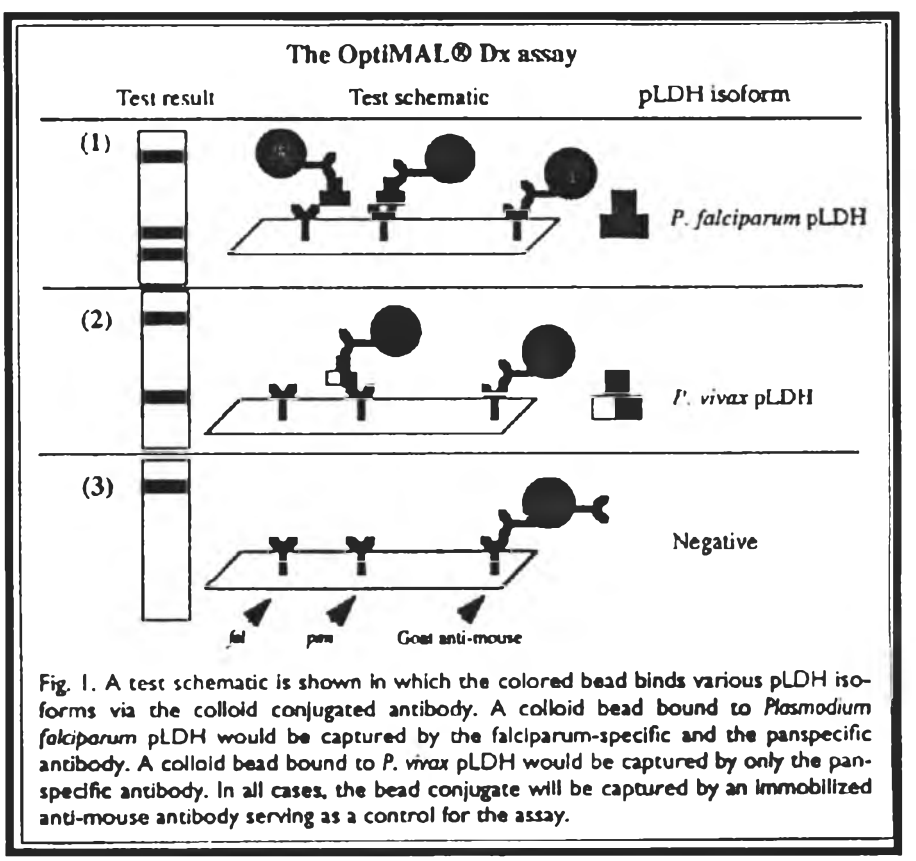


Fig. 1. A test schematic is shown in which the colored bead binds various pLDH isoforms via the colloid conjugated antibody. A colloid bead bound to *Plasmodium falciparum* pLDH would be captured by the falciparum-specific and the panspecific antibody. A colloid bead bound to *P. vivax* pLDH would be captured by only the panspecific antibody. In all cases, the bead conjugate will be captured by an immobilized anti-mouse antibody serving as a control for the assay.

Figure 2: Schematic diagram of the first OptiMAL assay, based on the detection of lactate dehydrogenase from the parasites (pLDH). Antibodies specific for the enzyme from *Plasmodium falciparum* (Fal) or pan-specific for all *Plasmodium spp.*

infecting man (Pan) and goat, anti-mouse antibodies (as a positive control) are immobilized in strips on the dipsticks. Three results are possible in a valid test:

(a) one control band plus two test bands, indicating the presence of *P. falciparum*;

(b) one control band plus one band, indicating the presence of *Plasmodium species* other than *P. falciparum*; or

(c) one control band only, indicating that no (viable) *Plasmodium spp.* were present in the blood sample.

Compared to microscopy, the antigen detection test or rapid tests offer both advantages and disadvantages as follow:

Advantages of rapid tests over microscopy

- Simpler to perform. Health worker with minimal skills can perform the test in a short time, as they require little training.
- Quick to perform. They are the tools of choice in malaria outbreak because they are easy to diagnose for treating a lot of people in a short period of time.
- Logistically easy to implement and maintain.
- Since the test detect antigen that release from *Plasmodium* parasites, so it can detect this substance when *P.falciparum* move to the deeper organ and cannot detect by malaria slide e.g. brain, spleen and placenta.

Disadvantages of rapid tests

- The tests are not quantitative (unlike microscopy): they cannot provide information regarding parasites density and parasites stages of development. Therefore they are not suitable for antimalarial drugs studies (follow-up studies).
- The test that detects both *P. falciparum* and *non-falciparum* cannot distinguish between *P.vivax, P.ovale* and *P.malariae* and cannot diagnose mixed infection as well.
- Interpretation of a result can be difficult. Each test has its own limitations, which should be assessed and understood before implementation.
- The current price range is US$ 0.5-3 per test; even at US$0.30-0.50 per test, these assays would not be affordable for most malaria control programs without external assistance.

The rationale of the study

To assess the new formulation of OptiMAL tests (OptiMAL-IT test) for the purpose of replace or supplement the microscopy for malaria diagnosis in rural areas as initial diagnosis test for malaria in fever presenting patients suspected malaria.

The review of the literatures

In the past several years, there were many studies conducted to evaluate the validity of OptiMAL test. Every study evaluated OptiMAL against microscopy as a gold standard, which in some studies there were only thick blood films and in some ones there were both thick and thin blood films were examined.

I would categorize the OptiMAL studies into four groups by using the objectives of the study as a criteria;

I) To evaluate the performance of the test as initial diagnostic study. These studies are Carol J. Palmer et al., 1998, A.H.Moody et al., 1998, Quintana M. et al., 1998, P. Bualombai et al., 1999, Hunt Cooke A. et al., 1999, Jelinek T. et al., 1999

II) To evaluate the performance of the test as antimalarial treatment monitoring tools. These studies are A.M.J. Oduola et al., 1997, C J Palmer et al., 1999.

III) To evaluate the performance of the test in both of initial diagnostic and antimalarial treatment monitoring tools. These studies are Moody A. et al., 2000, and S.Srivanasan et al., 2000.

IIII) To evaluate the performance of the test as epidemiological screening tool in asymptomatic population. There is only the study conducted by David J. Fryauff et al., 2000.

In the studies that purposes to evaluate the validity of OptiMAL assay as initial diagnostic, Carol J. Palmer et al., 1998 conducted a trial during a malaria outbreak in Honduras. A total of 202 patients with malaria like symptoms were included into this study that evaluate OptiMAL against microscopy. Unlike the other studies, 82% (79 of 96) of total cases in this trial are *P. vivax* and 18%(17 of 96) are *P. falciparum.* Mentioned in the study that OptiMAL can detect mixed infection by looking at the intensity of the band on dipstick, this statement later found it to be unlikely to rely. The results obtained were sensitivity 94% and 88% and specificity 100% and 99%

respectively, when compared to traditional blood films for the detection of *P. vivax* and *P. falciparum* infections. Sensitivity in detecting *P. vivax* was only 40% when parasites were present at less than 100μL of blood. The study of A.H.Moody et al., 1998 conducted in Tamil Nadu, India evaluated the first version of OptiMAL in 101 samples that stored at -70°C for up to six months against microscopy thick and thin blood films. The results demonstrated the sensitivity of 94% in *P. falciparum* infections and 98% in *P.vivax* infections. In Quintina M. et al., 1998 conducted in Honduran population. Two groups of studies were designed for hospital samples (symptomatic group) and field samples (asymptomatic group). The former group had average parasitaemia level approximately 591 parasites/ cu.mm, the latter had around 167 parasites/ cu.mm. The sensitivity of the test was 100% for parasites densities > 88 parasites/ cu.mm but decreased significantly at the level < 88 /cu.mm. In the pilot study that conducted in Thailand by P. Bualombai et al., 1999, which only thick blood film was used as a gold standard found OptiMAL provided a sensitivity and specificity level as good as microscopist performing in the field. However, *P. falciparum* infections in this study were only 14 cases and *P. vivax* infections were 10 cases. The Gambia study by Hunt Cooke A. et al., 1999 demonstrated the sensitivity of 100% at parasites densities ≥ 0.01% (500 parasites/μL) but decreased remarkably below this level, which is a disadvantage in using the test in non-immune population. Interestingly, the false negative from prozone phenomena was occurred in Jelinek T. et al 1999 study, which conducted in non-immune German travelers returning from malarious areas. A prospective multi-center study aimed to evaluate HRP-2 dipstick (ICT Pf) and pLDH assay (OptiMAL) against microscopy. The results obtained from both dipsticks were good. ICT Malaria Pf performed with 92.5% sensitivity and a specificity of 98.3%.

Compared to OptiMAL showed a sensitivity of 88.7% and a specificity of 99.4% Whereas the samples used to evaluate *P. vivax* infections and OptiMAL results was too small (13 out of 231 patients found *P. vivax* confirmed by microscopy). One sample with a very high parasitaemia of 20,000/μL was repeatedly negative in both dipsticks. Therefore, it should be emphasized that *P. falciparum* malaria, a potential lethal disease, must not be missed because of a false negative dipstick test.

In the studies that purpose to appraise the validity of OptiMAL assay as antimalarial treatment monitoring, A.M.J. Oduola et al., 1997 conducted in Nigeria was among the early studies about LDH activities in malaria infections. This study measured LDH level in blood products (whole blood, packed cell and plasma) by using Malstat™ reagent and an immunoenzymatic capture technique, which is quantitative. The results showed a consistent relationship between malaria infection and LDH activities that could be measurable in whole blood and packed cell but not in plasma. The study by C.J. Palmer et al., 1999 done in Guyana, assessed a short-term therapeutic response by using OptiMAL assay. Twelve cases of *P. falciparum* infections were appraised for 5 days post-treatment. By compared OptiMAL with microscopy both thick and thin smears, level of agreement of the two methods were 100% on day 0, declined to 45% on day 3 and was 83% on day 5. This findings probably because parasites that were seen in microscope may not be viable to produce pLDH enzyme. Therefore the OptiMAL became negative in spite of microscopy positive.

In the studies that assess the performance of the test in both of initial diagnostic and antimalarial treatment monitoring tools. The study organized at the Hospital for

Tropical Diseases, London by Moody A. et al., 2000. In an initial diagnosis reported a sensitivity of 95% and a specificity of 100% in *P. falciparum* infections. For *P. vivax* infections a sensitivity of 96% and a specificity of 100% was obtained. The ability to follow the course of parasitaemia during treatment by using OptiMAL is practically beneficial. The study found that the sensitivity of the test is lower after the starting of treatment than it would be before treatment at the same parasite densities. This may be because OptiMAL detects pLDH produced by viable parasites so its level declines in sequential samples. S.Srinivasan et al., 2000 compared blood-film microscopy, the OptiMAL, Rhodamine 123 fluorescence staining, which detects only the viable parasites and PCR in patients admitted in the Hospital of Tropical Diseases, London. A sensitivity of 100% in *P. falciparum* infections from initial blood samples and also the ability of OptiMAL that response to the decline in viability of the parasites during treatment were obtained from the study.

The only study that assessed the performance of the test as epidemiological screening tool in asymptomatic population was conducted in Irian Jaya, Indonesia by David J. Fryauff et al., 2000. The assay was performed in life-long malaria exposure population and short-term exposure population. Therefore, specificity achieved from the study was high with 97% and 89% respectively whereas sensitivity was 60% and 70% respectively. The OptiMAL false negatives were related to low parasitaemia (< 500/μL). From this cause, OptiMAL is unsuited for use as an epidemiological screening tool.

Conclusion

As recommended by D. PAYNE et al., 1988 that the new diagnostic technique, which simple, reliable and inexpensive should be emphasis. So they can be used even in the periphery of the primary health care system and provide the best possible guidance for the treatment of malaria in endemic areas.

This study will evaluate the new formulation of OptiMAL test (OptiMAL-IT), which has never been conducted yet. The ability to be used as initial diagnosis whether the test can be one of the alternatives in malaria diagnosis especially in the rural areas or the periphery of the primary health care system where microscopy is difficult to implement and maintain.

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