จุฬาลงกรณ์มหาวิทยาลัย
ทุนวิจัยสู่ชุมชน
กองทุนรัชดาภิเษกสมโภช

รายงานวิจัย

การประเมินคุณภาพการวินิจฉัยการตรวจมาลาเรีย โดยวิธีกล้องจุลทรรศน์ในคลินิก อำเภอแม่สอด จังหวัดตาก บริเวณชายแดนไทยพม่า

โดย

รัตนา สำโรงทอง

เมษายน ๒๕๕๓

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บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์ 1) เพื่อบรรยายลักษณะและเปรียบเทียบประสิทธิผลของการตรวจวินิจฉัยโรคมาลาเรียด้วยกล้องจุลทรรศน์ของเจ้าหน้าที่คลินิกชายแดนไทย-พม่ากับการวินิจฉัยของผู้เชี่ยวชาญและการใช้เทคนิคโพลีเมอเรสเชนรีแอคชั่น (พีซีอาร์) และ 2) เพื่อกำหนดแนวทางที่เป็นไปได้ในการปรับปรุงคุณภาพการตรวจวินิจฉัยโรคมาลาเรียของคลินิกในพื้นที่

วิธีการดำเนินการวิจัย: เป็นการศึกษาเปรียบเทียบการควบคุมคุณภาพการวินิจฉัยโรคมาลาเรียของคลินิกในชุมชน โดยชุมชนมีส่วนร่วม เพื่อประเมินการวินิจฉัยโรคมาลาเรียของคลินิกแม่ตาว โดยเก็บตัวอย่างเลือดผู้ป่วย768 ราย พร้อมทำแผ่นฟิล์มโลหิตหนาและบาง เจ้าหน้าที่ของคลินิกอ่านผลแผ่นฟิล์มโลหิตโดยใช้กล้องจุลทรรศน์ แผ่นฟิล์มโลหิตและตัวอย่างเลือดจากผู้ป่วยจำนวน 339 ราย ถูกสุ่มเพื่อส่งตรวจยังห้องปฏิบัติการในกรุงเทพฯ แผ่นฟิล์มโลหิตจะถูกอ่านผลโดยผู้เชี่ยวชาญจากกระทรวงสาธารณสุข ตัวอย่างเลือดจะใช้ในการตรวจวิเคราะห์ด้วยวิธีพีซีอาร์ ในการอ่านผลผู้อ่านผลจะไม่ทราบผลที่ได้จากหน่วยงานอื่นๆ ความไวและความจำเพาะของการตรวจวินิจฉัยด้วยกล้องจุลทรรศน์ทั้งสองกลุ่มถูกคำนวณเทียบกับผลที่ได้จากวิธีพีซีอาร์ ผลการวินิจฉัยชนิดเชื้อมาลาเรียของแต่ละกลุ่มจะเปรียบเทียบโดยใช้วิธีทางสถิติ (kappa statistic)

ผลการวิจัย: ความไวและความจำเพาะของการวินิจฉัยโรคมาลาเรียของคลินิกแม่ตาวเท่ากับ 90.5% (95%CI, 85.8-95.2) และ 98.9% (95%CI, 97.4-100.0) สำหรับการวินิจฉัยโดยผู้เชี่ยวชาญกระทรวงสาธารณสุขมีความไวเท่ากับ 93.2% (95% CI, 89.2-97.2) และความจำเพาะเท่ากับ 98.9% (95% CI, 97.4-100.0) ผลการวินิจฉัยโรคที่ผิดพลาดของคลินิกแม่ตาวทำให้ผู้ป่วยติดเชื้อมาลาเรียชนิดฟัลซิพารัมจำนวน 13 ราย และผู้ป่วยติดเชื้อมาลาเรียชนิดไวแวกซ์ 8 รายไม่ได้รับการรักษาที่ถูกต้อง ในขณะที่ผลการวินิจฉัยที่ผิดพลาดของผู้เชี่ยวชาญจากกระทรวงสาธารณสุขทำให้ผู้ป่วยติดเชื้อมาลาเรียชนิดฟัลซิพารัม 10 รายและผู้ป่วยติดเชื้อมาลาเรียชนิดไวแวกซ์ 5 รายไม่ได้รับการรักษาที่ถูกต้อง วิเคราะห์ความตรงกันของผลการวินิจฉัยจากทั้งสามหน่วยงานด้วยวิธีทางสถิติพบว่าการวินิจฉัยของคลินิกแม่ตาวกับผลพีซีอาร์มีความตรงกัน 0.687 (95% CI, 0.573-0.801) การวินิจฉัยของผู้เชี่ยวชาญจากกระทรวงสาธารณสุขกับผลพีซีอาร์มีความตรงกัน 0.868 (95% CI, 0.789-0.947) และการวินิจฉัยของเจ้าหน้าที่คลินิกแม่ตาวเปรียบเทียบกับการวินิจฉัยของผู้เชี่ยวชาญจากกระทรวงสาธารณสุขมีความตรงกัน 0.737 (95% CI, 0.628-0.845) ผู้เชี่ยวชาญจาก

กระทรวงสาธารณสุขมีความเห็นให้เจ้าหน้าที่คลินิกแม่ตาวทำการปรับปรุงคุณภาพการทำฟิล์มโลหิตหนาและบางเนื่องจากมีฟิล์มโลหิตหนา 60.8% และฟิล์มโลหิตบาง 41.0% ที่ไม่ได้คุณภาพ

คลินิกแม่ตาวมีกล้องจุลทรรศน์ที่มีประสิทธิภาพในสภาพที่ทรัพยากรจำกัด การวินิจฉัยผู้ติดเชื้อมาลาเรียมีความถูกต้องเทียบเท่ากับเจ้าหน้าที่จากกระทรวงสาธารณสุข แต่เจ้าหน้าที่คลินิกแม่ตาวจำแนกชนิดของเชื้อมาลาเรียผิดพลาดมากกว่า การปรับปรุงเทคนิคการทำฟิล์มโลหิตและการย้อมสี การควบคุมคุณภาพในการวินิจฉัยเป็นประจำ ตรวจสอบให้มีการปฏิบัติงานได้ตามมาตรฐานที่ดีอยู่เสมอจะช่วยเสริมให้ความสามารถในการวินิจฉัยโรคมาลาเรียในบริเวณชายแดนไทย-พม่ามีประสิทธิภาพ

Project Title: Quality Assessment of Malaria Diagnosis by Microscopy at Clinic in Mae Sot District, ak Province, on the Thai-Burma Border

Principle Investigator: Assist. Prof. Dr. Ratana Somrongthong

Project Ended Date: April, 2010

Abstract

This study aims to 1) characterize and compare effectiveness of malaria diagnosis by microscopy at a Thai-Burma border field clinic with that of expert microscopy diagnosis and PCR and 2) Identify potential ways to improve field clinic malaria diagnosis quality.

Methods: A prospective community-based participatory research cohort quality control study was performed to assess Mae Tao Clinic malaria diagnostic performance. Between March 5-31, 2007, clinic staff collected blood samples and obtained thin/thick blood smears from 768 consecutive patients meeting inclusion/exclusion criteria and providing informed consent. Clinic microscopists interpreted blood smears and treated patients per clinic protocol. Blood smears and samples from 339 randomly-selected subjects were sent to reference laboratories. Blood smears were interpreted by expert MoPH microscopists; PCR was performed on blood samples. All interpretations were blinded to other results. Sensitivity and specificity were calculated for both microscopist groups, using PCR results as reference standard. Diagnostic performance among groups was compared using kappa statistic.

Results: Malaria diagnosis sensitivity and specificity respectively were 90.5% (95% CI, 85.8-95.2) and 98.9% (95% CI, 97.4-100.0) for the clinic and 93.2% (95% CI, 89.2-97.2) and 98.9% (95% CI, 97.4-100.0) for the MoPH. Clinic diagnostic errors resulted in 13 untreated P. falciparum and 8 untreated P. vivax infections versus 10 untreated P. falciparum and 5 untreated P. vivax infections for the MoPH. Kappa agreement was 0.687 (95% CI 0.573-0.801) between clinic staff and PCR; 0.868, (95% CI 0.789-0.947) between MoPH staff and PCR; and 0.737 (95% CI 0.628-0.845) between clinic and MoPH staff. MoPH comments about blood smear quality indicated 60.8% of thick smears and 41.0% of thin smears needed improvement.

Conclusions: The Mae Tao Clinic has implemented effective malaria microscopy in a resource-limited field setting. Diagnostic accuracy for malaria infection was comparable for clinic and MoPH staff, but clinic staff made more species-specific errors. Improvements in blood smear staining and smearing techniques, regular quality control studies, and best-practice standard monitoring may further enhance Thai-Burma border field diagnostic performance.

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Introduction

While the overall malaria burden in Thailand has declined rather dramatically over the past 20 years, the disease remains an important public health problem along the Thai-Burma border.[1] The northern province of Tak has been Thailand's most malarious region for the past decade and includes areas with the highest prevalence of multidrug-resistant *Plasmodium falciparum* parasites in the world.[1, 2, 3] The high volume of cross-border migration in this area over the past few decades, placed against the backdrop of political instability in Burma, provides the driving force behind these epidemiologic trends.[3] Large numbers of migrants fleeing civil war in Burma who seek labor and refuge in Thailand often spend substantial amounts of time in malaria-infested forests, where health services and essential medications are largely unavailable.[4] Given the extensive problem of drug resistance, accurate and timely malaria diagnosis is essential in order to initiate appropriate, species-specific drug treatment. Nowhere is this concept more important than in the field, where transient patients with very few resources may only have one chance to visit a health care facility, and treatment decisions must be made at the time of presentation and initial evaluation.

The Mae Tao Clinic, located in the border town of Mae Sot, Tak province, is a field site that provides health care services to hundreds of thousands of internally displaced persons who cross the border from Burma, as well as mostly Burmese migrants living in Mae Sot[5]. Since the clinic must give urgent medical care to vulnerable patient populations in a resource-limited setting, a major challenge is trying to adhere as much as possible to the "best practice" standards of care while lacking many of the medications, technologies, and human resources available under more ideal conditions. The clinic attempts to meet this challenge by offering

the most highly recommended and updated treatments available within its means. As an example, its protocol for malaria diagnosis and treatment follows WHO guidelines for Thailand and the rest of Southeast Asia, which includes clinical diagnosis confirmed by microscopy and first-line treatment for *P. falciparum* consisting of 3 days of artesunate and mefloquine (MA3)[6*].

In order to ensure that the Mae Tao Clinic meets best practice standards for malaria diagnosis, its microscopy facilities should undergo quality control review at regular intervals, ideally by a qualified external agency. Mae Tao microscopists must detect malaria with high sensitivity in order to avoid missed infections, particularly *P. falciparum*, the most virulent of the malaria species, which requires a specific drug regimen. High specificity is also desirable in order to prevent unnecessary use of medications for *P. falciparum* that could promote drug resistance and incur extraneous expense. In the past, a well-established, local NGO performed quality control for the clinic on two occasions, but since September 2006, the clinic has been conducting its own internal review.[7, 8]

To address the current need for external quality control analysis, a blinded study assessing the accuracy of Mae Tao Clinic malaria diagnosis by microscopy was designed, implemented, and analyzed. While microscopy is the gold standard diagnostic method in the field, polymerase chain reaction (PCR) is a molecular-based test that can detect malaria parasites with higher sensitivity than microscopy and identify mixed infections (e.g. *P. falciparum* + *P. vivax*) that may be missed by slide diagnosis alone.[9] Though PCR is not feasible in the field due to time, facility, and financial constraints, it provides an appropriate reference standard with which to evaluate Mae Tao Clinic microscopists' performance. To help place the Mae

* Fortunately, the three remaining malaria species, *P. vivax, P. ovale,* and *P. malariae*, are still largely susceptible to chloroquine and primaquine in this region[19].

Tao Clinic staff's diagnostic accuracy in an appropriate context, their performance was also compared to that of expert microscopists from the Thai Ministry of Public Health Laboratory for Vector-Borne Disease Control (Bangkok, Thailand), who perform quality control analysis for all government hospitals, clinics, and health posts throughout Thailand. By cross-checking both groups' results with PCR performed at the Malaria Unit, Institute of Health Research, Chulalongkorn University (Bangkok, Thailand), we aimed to compare the micrsocopy diagnostic performance between Mae Tao Clinic microscopists and Ministry of Public Health microscopists in order to assess whether the Mae Tao Clinic was indeed meeting the best practice standards attainable.

Methods

Study Design

Between March 5 and May 16, 2007, a prospective, community-based participatory research cohort quality control study was performed to assess the diagnostic accuracy of malaria microscopists at the Mae Tao Clinic in Mae Sot, Thailand.

Selection and Description of Participants

Study participants were clinic outpatients five years of age or older, including pregnant women, who presented to the clinic as new cases with fever and were referred to the clinic lab for malaria testing. Exclusion criteria were age less than five years, anti-malarial treatment and/or previous blood smears taken during the current clinic visit, and inability to understand the informed consent proceedings when conducted in the Burmese language.[†]

Verbal and written explanations of the study protocol were provided in the Burmese language, and each study participant or his/her guardian provided written informed consent with a signature or X (if the participant was unable to read or write). Subjects were informed of the risks and benefits of study participation and told that study participation was voluntary, that consenting participants would be giving two small blood samples, and that their personal identification information and test results would be kept confidential, The study was approved by the Chulalongkorn University Health Science Faculties Ethical Review Committee for Research Involving Human Subjects (Bangkok, Thailand).

[†] Most clinic patients, though hailing from diverse ethnic backgrounds (including Karen, Shan, Kachin, and Arakan), understand the Burmese language, which is used as a lingua franca among most Burmese people. However, some patients, especially those from more rural areas of Burma, only speak the language of their particular ethnic group.

Sample Collection

From March 5-31, 2007, patients with suspected malaria who presented Mae Tao Clinic medics were referred to the Mae Tao Clinic laboratory for testing. Mae Tao Clinic laboratory microscopists collected a 0.5 ml fingerprick blood sample for PCR analysis from each patient on a strip of filter paper (Whatman, Kent, UK) labeled with the patient's unique identification code. After air-drying, PCR samples were placed in individual plastic bags and stored in an airtight, closed container until the end of the four-week sample collection period.

Thin and thick blood smears were then obtained from each patient's fingerprick site, labeled with their identification code, stained with Giemsa, and read under a light microscope.

Sample Analysis

Based on Mae Tao Clinic diagnoses of the blood smears, patients were treated per normal clinic protocol (3 days of Artesunate + Mefloquine for uncomplicated *P. falciparum* single or mixed infection; 3 days of Chloroquine + Primaquine for uncomplicated *P. vivax, P. malariae,* or *P. ovale* single or mixed infection)[6]. All blood smears taken over the 4-week sample-collection period were separated into "malaria positive" or "malaria negative" categories after initial reading and stored in corresponding slide boxes until the end of the sample-collection period.

Following sample collection, all of the "malaria positive" slides and a randomly-selected subset of "malaria negative" slides were chosen for inclusion in the final quality control study analysis (see "Statistical Analysis" section for more details). These positive and negative slides were mixed together, placed in chronological order of when they were collected, and transported to Bangkok for microscopic analysis at the Thai Ministry of Public Health. Subsequently, PCR samples corresponding to the blood smears selected for quality control

analysis were identified, separated, and transported to Bangkok for PCR analysis at the Malaria Unit, Institute of Health Research, Chulalongkorn University.

Microscopy

From April 5-May 4, 2007, expert microscopists from the Thai Ministry of Public Health [MoPH], blinded to Mae Tao Clinic results, examined and interpreted the blood smears transported from the Mae Tao Clinic [MTC]. Any blood smears for which MoPH results were discordant with MTC results (as determined by the principal investigator), were read a second time by a different MoPH microscopist who was aware that the initial results were discordant but did not know the nature of the discordant results. The second reading was taken as the final MoPH result.

Slide Quality

In addition to interpreting MTC blood smears, the MoPH was also asked to provide comments about thick smear quality, thick smear color, and thin smear quality. In order to evaluate the quality and color of each smear, a three-tiered rating scale was developed (Table 1). When determining which comments to ascribe to a slide, MoPH microscopists evaluated certain descriptive criteria (outlined in the "Smear Quality Description" and "Smear Color Description" boxes) as part of their protocol. Based on how well these criteria were met, a comment for each of the three categories was given. These comments (outlined in the "MoPH Comments" boxes) were then grouped into a 3-tiered rating scale ("Good," "Average," and "Poor") based on their subjective significance. Each slide then received one of these three grades for each of the three parameters described.

Table 1: Rating scale for evaluating smear quality and color

	- Standard - Good - Very good	- Average - Usable	- Below standard - Needs improvement - Poor - Too thin - Too thick
	- Appropriate thickness - Covers enough area (1 cm^2) - Little to no Giemsa residue - Little to no artifact - For thick smears, 10-20 WBC per field	- Moderate Giemsa residue - Moderate artifact - Does not cover appropriate area - For thick smears, 5-10 WBC per field	- Too much artifact - Too many precipitins - Too much or too little blood - Blood film spread unevenly - Blood rubbed off - Autofixation - For thick smears, ≤ 5 WBC per field - Too much Giemsa residue/dust
	- Pinkish-blue color	- Blue color	- Too red - Too blue

DNA Extraction & Purification[†]

From April 12-May 16, 2007, DNA extraction and PCR analysis of the blood samples from Mae Tao Clinic were performed at the Malaria Unit, Institute of Health Research, Chulalongkorn University according to protocol outlined by Georges Snounou et al (1993)[10].

DNA extraction was done using Chelex® 100 [ion exchange] Resin (Bio-Rad, Hercules, California, USA). To prepare the resin for each sample, 50 µl of a 20% Chelex® 100 stock solution in water were diluted with 150 µl of deionized water, the mixture then vortexed and heated in a heating box (Barnstead/Thermolyne, Iowa, USA) at 100°C for 35 min.

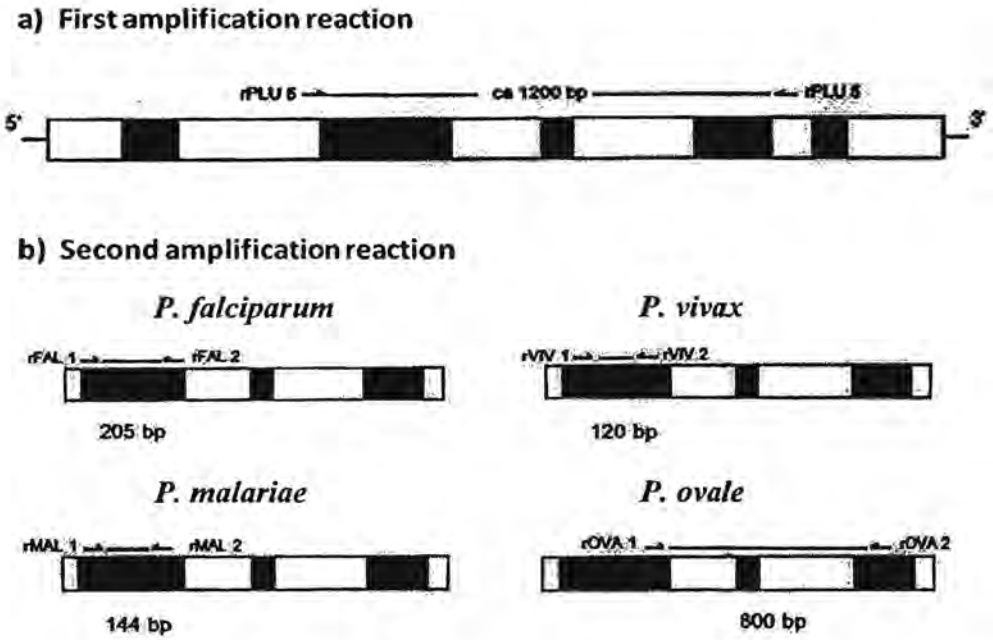
A 3 mm strip from each blood-saturated filter paper sample was cut and soaked in 1 ml phosphate-buffered saline (PBS) overnight at 4°C. Parasites and unlysed erythrocytes were pelleted on the paper strip by brief centrifugation (9 sec, "Quick Run" mode). The supernatant was discarded and the paper resuspended in 0.5 mL of 0.5% saponin in PBS solution. Samples were refrigerated for 10 min at 4°C while RBC lysis occurred. Parasites and white blood cells on the paper were recovered by centrifugation as above and the supernatant discarded. Each paper strip was rinsed with 1 ml PBS and centrifuged again as above, and the supernatant discarded. The paper was resuspended in 1 ml PBS and refrigerated for 30 min at 4°C to allow saponin washout. Each paper strip was extracted and immersed in 200 µl of the Chelex® 100 solution prepared in the first step. The paper and Chelex® 100 solution were vortexed for 30 sec and heated in the heating box at 100°C for 10 min. At the 5- min halfway point of heating, each sample was vortexed again for 10 sec and returned to the heating box. After the complete 10 min heating period, each sample was vortexed for 20 sec to allow DNA extraction from the paper into the solution. The paper and excess Chelex® 100 resin were separated from the DNA-containing supernatant via centrifugation (3 min, 13,000 RPM). The supernatant from each sample was then transferred to another tube and centrifuged again as above to allow precipitation of additional excess resin. The purified DNA-containing supernatant for each sample was then transferred to a final tube and stored at -20°C to be used as the DNA template for subsequent PCR amplification.

DNA templates used as positive controls were obtained from malaria parasites whose species identity had been confirmed by expert microscopy and PCR performed at the Malaria Unit, Institute of Health Research, Chulalongkorn University. The DNA was purified using the phenol-chloroform extraction protocol developed by Georges Snounou (1994).[11]

PCR Amplification

PCR amplification of Plasmodium DNA was performed using the nested PCR technique outlined by Georges Snounou et al (1993).[12] The protocol used to amplify the desired Plasmodium genes consisted of 2 reactions: 1) Amplification of the genus-specific Plasmodium gene using purified DNA from each sample as the DNA template; and 2) Amplification of each species-specific Plasmodium gene, using the DNA product from Reaction 1 as the DNA template. For the first reaction, the genus-specific primer pair rPLU5 and rPLU6 (Sigma Proligo, Singapore), was used to amplify the 1.2 kb gene coding for a Plasmodium ssrRNA sequence common to all Plasmodium species. For the second reaction, species-specific primer pairs (rFAL1/rFAL2, rVIV1/rVIV2, rMAL1/rMAL2, and rOVA1/rOVA2, Sigma Proligo, Singapore) were used to detect the presence of each Plasmodium species within the DNA product obtained in Reaction 1. This species-specific reaction was run separately for each sample. A specific ssrRNA gene product from Reaction 2 (205 bp for *P. falciparum*, 120 bp for *P. vivax*, 144 bp for *P. malariae*, and 800 bp for *P. ovale* as shown in Figure 1) was only obtained if DNA from the corresponding species was present in the reaction--i.e. a positive infection from that species in a given sample. Neither Reaction 1 nor Reaction 2 yielded any DNA product if there was no Plasmodium DNA in a given sample—i.e. a negative infection.

All PCR reactions were performed in a total volume of 20 µl. Each reaction was carried out using an AmpliBuffer (Vivantis, Chino, California, USA) containing 500 mM KCl, 100 M Tris-HCl (pH 9.1), and 0.1% Triton™ X-100; 2 mM $MgCl_2$; 125 µM of deoxyribonucleotide triphosphates; 250 nM of each oligonucleotide primer, and 0.44 units *Taq* DNA Polymerase (Vivantis, Chino, California, USA). For Reaction 1, 1 µl of purified DNA template from each sample was used to amplify the 1.2 kb genus-specific *Plasmodium* gene if present. For Reaction 2, a 1-µl aliquot of the DNA product obtained from Reaction 1 was used as the DNA template for each of the 4 separate reactions aimed to detect the species-specific *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* genes if present.



c) Amplified product on agarose gel

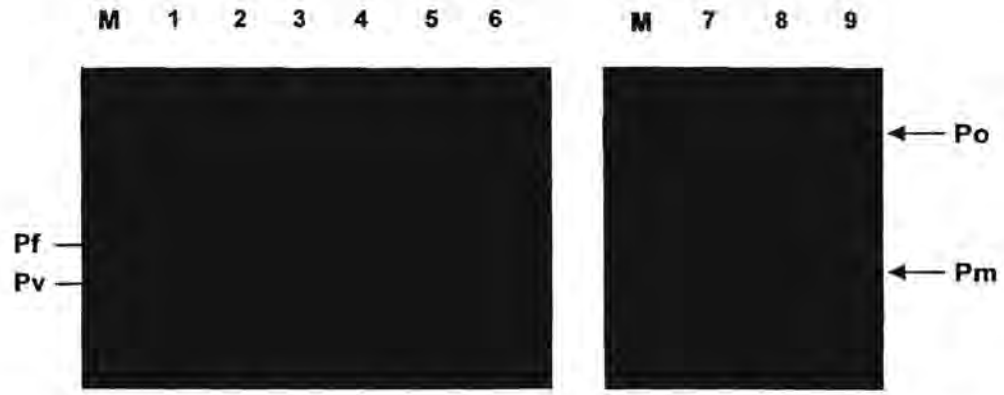


Figure 1: Schematic representation of *Plasmodium* ssrRNA genes; a) the first reaction for genus specific amplification, b) the second reaction for species specific amplification. The black boxes represent variable sequences unique to each species (Modified from Snounou et al., 1993), c) the PCR products were separated on agarose gels: M stand for 100 bp DNA ladder, lane 1 presented positive control for *P. falciparum* and *P. vivax*, lane 2-6 presented the PCR products from the samples, lane 7 presented positive control for *P. ovale* and *P.malariae*, lane 8-9 presented the PCR products from the samples.

For Reaction 1, the PCR amplification program parameters were as follows. Step 1: Initial denaturing at 95°C for 4 min; Step 2: Further denaturing at 95°C for 1 min; Step 3: Annealing at 58°C for 2 min; Step 4: Extension at 72°C, 2 min; Step 5: Final extension at 72°C, 2 min. Steps 2-4 were cycled 25 times in between steps 1 and 5. At the end of the program, the temperature was reduced to 20°C. For Reaction 2, the program parameters and cooling temperature remained the same, except steps 2-4 were cycled 30 times in between steps 1 and 5.

PCR Product Analysis

3% NuSieve agarose/Agarose (3:1) gels were made using 2.25 g NuSieve agarose (FMC BioProducts, Rockland, USA) and 0.75 g agarose (USB, Cleveland, USA) dissolved in 100 ml

1x TBE buffer (Tris, boric acid, EDTA pH 8.0), 1 µl loading dye, and 2 µl DNA product were loaded into each gel well, with a 100 kb marker and positive controls loaded into the first and second wells in each column, respectively. For most electrophoresis procedures, DNA products and positive controls from the *P. falciparum* and *P. vivax* species for each sample were loaded together in the same well, as were the DNA products and positive controls from the *P. malariae* and *P. ovale* species for each sample, in order to save time and conserve materials. The gels were immersed in 1x TBE buffer, and amplification products were electrophoresed at 70 mV. The gels were then stained in ethidium bromide and the DNA products visualized under ultraviolet light.

PCR technicians were blinded to MTC and MoPH results. Any samples for which PCR results disagreed with MTC or MoPH microscopy results (as determined by the principal investigator) underwent PCR a second time. If the second PCR result was discordant with the first PCR result, the sample underwent PCR a third time. PCR technicians were aware that previous results were discordant but did not know the nature of the discordant results. The final PCR result was considered the one that appeared 2 out of 3 times.

Statistical Analysis

Sample size was calculated based on a desired precision level of $\leq .03$ for a 95% confidence interval and expected sensitivity $\geq 95\%$, using the equation:

$$n = \frac{Z^2 p (1-p)}{L^2}$$

where n = sample size of positive *or* negative selected slides, Z = 1.96, p = estimated sensitivity (0.95), and L = desired precision level (0.03). From this equation, $202.6 \approx 203$ was

the desired sample size for positive *or* negative slides; therefore, 203 slides were randomly selected from the total number of 626 eligible negative slides (approximately 1 out of 3). Random selection was accomplished by extracting and keeping every 3rd slide from the "malaria negative" slide box without regard to any characterizing features such as perceived slide quality, date of smear collection, or patient identification information. Since the total number of eligible positive slides was only 136, all positive slides were included in study analysis in order to provide an adequate proportion of positive and negative samples.[13, 14]

Frequencies and cross-tabulations for MTC, MoPH, and PCR results were obtained using SPSS v. 13 for Windows (SPSS, Chicago, Illinois, USA). From these, the sensitivity, specificity, positive predictive value, and negative predictive value (± 95% confidence intervals) were calculated for the MTC and MoPH using standard formulas, with PCR as the reference standard, and again for the MTC using final MoPH interpretations as the reference standard. To assess what these quality indicators for the MoPH would have been had MoPH microscopists not had the opportunity to re-interpret discordant results, these four parameters were also calculated for the initial MoPH interpretation, using PCR as the reference standard. P-values were calculated to compare frequencies and quality indicators among all three groups, using an interactive chi-square calculation tool developed by Preacher (2001)[15]. Agreement of diagnostic performance among all three groups was compared using an unweighted kappa statistic. Interpretation of kappa values was based on the Altman (1991) classification.[16]

Possible results for malaria diagnosis included no infection ("negative"), a single-species infection (e.g. *P. falciparum*), or a mixed-species infection (e.g. *P. falciparum* + *P. vivax*). A microscopy result was considered "accurate" if it matched the PCR result exactly; therefore, if

a microscopist interpreted a sample as *P. falciparum* that was interpreted as *P. falciparum* + *P. vivax* by PCR, the microscopist's interpretation would not be considered accurate, even though one of the species present was correctly identified. Similarly, results were considered to be "in agreement" if they matched exactly; simply diagnosing one species correctly for a mixed infection was not sufficient to constitute agreement.

Errors made by MTC and MoPH microscopists were described, quantified, and correlated with the perceived quality of the misdiagnosed slide. P-values were calculated to compare error counts for the MTC and MoPH.

Since the above summary statistics are less important than the clinical implications for the patients to which they refer, MTC and MoPH errors resulting in incorrect therapy for *P. falciparum* and/or *P. vivax* were identified. To examine the clinical consequences of an inaccurate diagnosis, patients who did not receive adequate or appropriate treatment as a result of these errors were followed up to see whether they ever were properly diagnosed and treated at a subsequent clinic visit.

Results

Sample Selection

Of 2228 total blood smears taken by Mae Tao Clinic staff between March 5-31, 2007, 845 came from eligible patients. Of these patients, 786 consented to participate in the study. Of these, 762 had blood smears and PCR samples recovered from them successfully. Of the 762 smears, 136 were interpreted as positive, and 626 were interpreted as negative by Mae Tao Clinic staff. All 136 positive smears, (100.0%) were submitted for quality control analysis. Of the 626 negative smears, 203 (32.4%) were randomly selected for quality control analysis. Therefore, a total of 339 blood smears and their corresponding PCR samples were included in the quality control analysis (Figure 2).

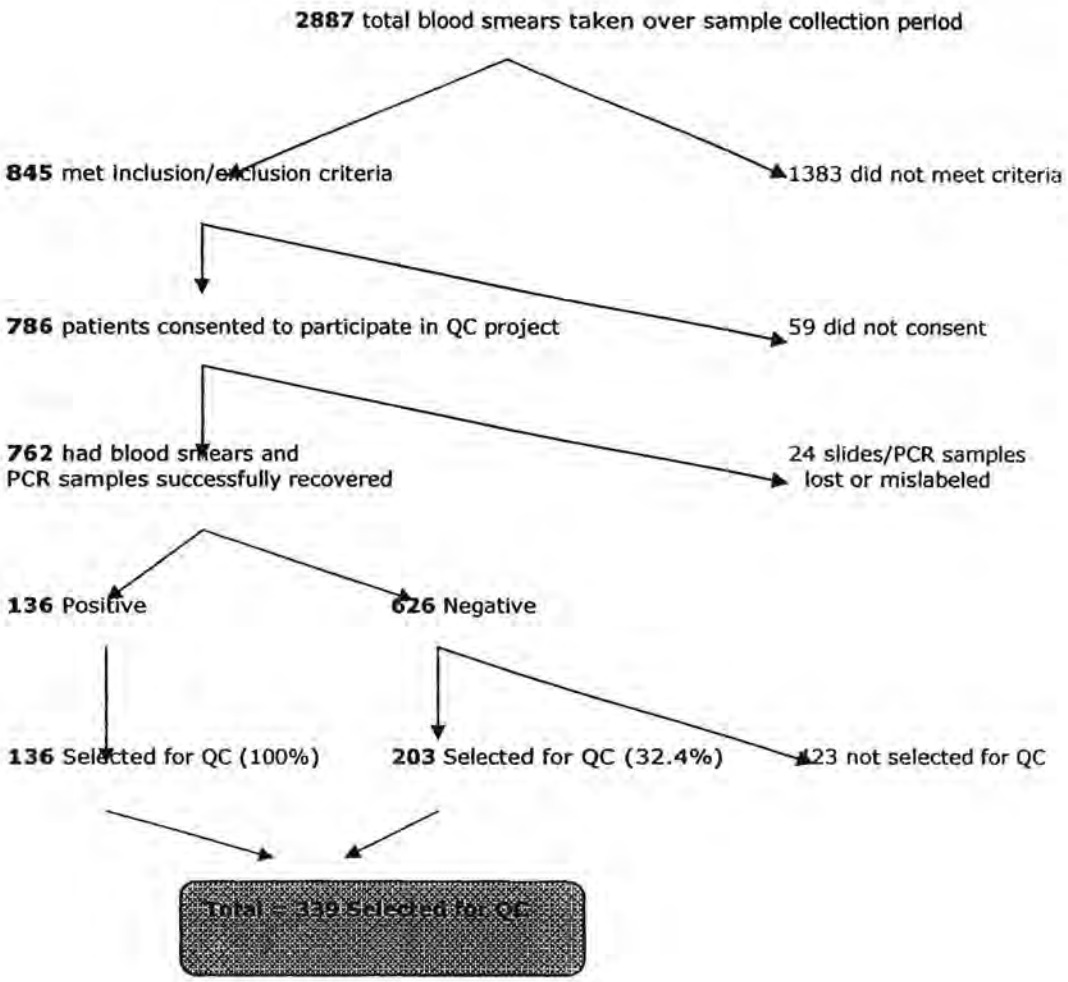


Figure 2: Flow chart of sample selection

Sample Concordance and Errors

A summary of frequencies for each of the three diagnostic modalities (MTC microscopy, MoPH microscopy, PCR) can be found in Table 2. There was high concordance in sample interpretation among Mae Tao Clinic staff, Ministry of Public staff, and PCR results ($p = 0.48$). More detailed frequency tables are provided in Appendix 1. Even though the final MoPH and final PCR interpretations were the results used for statistical analysis, it is worth noting that MoPH staff had the opportunity to re-evaluate 38 slides that were originally discordant with MTC results, while 91 PCR samples with interpretations originally discordant with microscopy (27% of total samples) underwent PCR amplification a second time, and 49 PCR samples (12.1% of total samples) with interpretations discordant with previous PCR results underwent amplification a third time.

Table 2: *Plasmodium* species identification of all 339 selected blood samples

Dx Method	Neg	Pf	Pv	Pf + Pv	Pf + Pv + Pm	Pf + Pv + Po	Pm	Po
MTC Microscopy	**203** 59.9%	**67** 19.8%	**59** 17.4%	**9** 2.7%	**0**	**0**	**1** 0.3%	**0**
MoPH Microscopy	**199** 58.7%	**64** 18.9%	**73** 21.5%	**3** 0.9%	**0**	**0**	**0**	**0**
PCR	**191** 56.3%	**65** 19.2%	**71** 20.9%	**9** 2.7%	**1** 0.3%	**1** 0.3%	**0**	**1** 0.3%

MTC = Mae Tao Clinic microscopy interpretation

MoPH = Ministry of Public Health final microscopy interpretation

PCR = polymerase chain reaction final interpretation

***Neg** = negative; **Pf** = Plasmodium falciparum; **Pv** = Plasmodium vivax; **Pm** = Plasmodium malariae; **Po** = Plasmodium ovale*

Overall, 40 out of 339 (11.8%) MTC results cross-checked with PCR were incorrect, compared with 22 (6.5%) of MoPH results that were incorrect ($p = 0.02$) Of the 40 MTC errors, 16 were false-negative or false-positive (negative-positive errors) and 24 were species-specific errors (e.g. *P. falciparum* misdiagnosed as *P. vivax*). In comparison, the MoPH made 12 negative-positive errors and 10 species-specific errors. There were a total of 11 mixed infections detected by PCR (7.4% of all 148 positive infections detected by PCR). The Mae Tao Clinic misdiagnosed 7 (63.6%) of these infections as either negative or a single infection ($p = 0.001$ for MTC vs. PCR). In comparison, the MoPH misdiagnosed 8 (72.7%) of these infections as either negative or a single infection ($p = 0.0004$ for MoPH vs. PCR; $p = 0.64$ for MTC vs. MoPH). A summary of the different types of errors made by both groups of microscopists can be found in Table 3.

Table 3: Types of Errors (compared with final PCR interpretation)

Diagnostic Method	Total # Neg/Positive Errors	Total # Species-Specific Errors	Total # missed mixed infections (out of 11)	Total # Errors (out of 339)
MTC Microscopy	16	24	7 (63.6%)	40 (11.8%)
MoPH Microscopy	12	10	8 (72.7%)	22 (6.5%)

Clinical Consequences of Errors

As a result of Mae Tao Clinic diagnostic errors, 13 of 76 *P. falciparum* (*Pf*) cases (17.6%) went untreated, and 8 of 82 *P. vivax* (*Pv*) cases (9.8%) went untreated. 13 of 263 patients (4.9%) received unnecessary treatment for *Pf*, and one patient out of 257 (0.4%) received unnecessary treatment for *Pv*. In comparison, if treatment outcomes had been dictated by MoPH diagnostics, MoPH errors would have resulted in 10 untreated *P. falciparum* cases

(13.1%), 5 untreated *P. vivax* cases (6.1%), one patient unnecessarily treated for *Pf* (0.3%) and 2 patients unnecessarily treated for *Pv* (7.8%). (Comparing consequences of MTC errors versus MoPH errors, $p = 0.50$ for missed *Pf*; $p = 0.39$ for missed *Pv*; $p = 0.001$ for unnecessary *Pf* treatment; $p = 0.56$ for unnecessary *Pv* treatment). Please see Table 4 for more details.

Table 4: Clinical Consequences of Errors

Diagnostic Method	Untreated Pf Cases (out of 76 Pf)	Unnecessary treatment of Pf (out of 263 non-Pf)	Untreated Pv Cases (out of 82 Pv)	Unnecessary treatment of Pv (out of 257 non-Pv)
[illegible]	**13** (17.6%)	**13** (4.9%)	**8** (9.8%)	**1** (0.4%)
MoPH [illegible]	**10** (13.1%)	**1** (0.3%)	**5** (6.1%)	**2** (7.8%)

Of the 20 patients who went untreated due to MTC diagnostic errors, 12 had untreated *Pf*, 7 had untreated *Pv*, and one had an untreated mixed *Pf* + *Pv* infection.[§] A summary of the follow-up and treatment profiles of these patients can be found in Table 5.

Of the untreated *P. falciparum* cases, seven patients (#'s 2, 3, 5, 7, 12, 17, 19) never received appropriate treatment for their infections; three (#'s 9, 15, 18) presented again to the clinic later that week, were correctly diagnosed with *Pf* the second time, and received appropriate treatment; and two untreated *Pf* patients (#'s 13, 14) were mistakenly diagnosed and treated for *P. vivax*, but their follow-up visits to the clinic later that week showed negative blood smears. One patient (#8) with a mixed *Pf* + *Pv* infection was treated for *Pv* only, but his

[§] One Pf patient (#'s 3 and 5 in Table 4) who had a negative smear returned to the clinic 4 days later, had another negative smear, and therefore did not receive any malaria treatment. Since both his visits fell within the sample-collection period and both his smears were randomly selected for QC analysis, he is counted as 2 patients for the purposes of this study.

follow-up visit later in the week showed a negative smear. Of the untreated *P. vivax* cases, 6 patients (#'s 1, 4, 7, 11, 16, 20) never received appropriate treatment for their infections. One untreated *Pv* patient (#6) presented to the clinic later that week, was diagnosed with *Pf*, and received appropriate treatment for *Pf*, which should have eradicated the *Pv* infection. One untreated *Pv* patient (#10) presented again to the clinic later that week, was correctly diagnosed with *Pv* the second time, and received appropriate treatment. Two triple infections (*Pf+Pv+Pm* and *Pf+Pv+Po*) were present among this patient group, but the *P. malariae* and *P. ovale* infections are not considered separately in the results analysis because there was only one case of *Pm* and 2 cases of *Po*, and treatment for these species is the same as for *Pv*. The *Pm* infection (while misdiagnosed as *Pv* only) was adequately treated with the *Pv* regimen, whereas the *Po* infection was untreated.

Table 5: Treatment and follow-up profiles for missed malaria cases

ient #	Age	Sex	MTC Result	MoPH result	PCR Result	Treatment Outcome
	20	F*	Neg	Pv	Pv	No malaria tx; presented again 30 d later w/ fever/headache/dysuria → neg smear, tx for UTI. Delivered healthy 2.4 kg baby 69 d later.
	12	M	Pm, g	Pv	Pf + Pv + Pm	Tx for Pv and Pm only. No follow-up.
	35	M	Neg	Pf	Pf	No malaria tx; presented 4 d later w/ 103 F fever and neg smear → dx w/ appendicitis. No planned follow-up.
	7	F	Neg	Pv	Pv	No malaria tx. No follow-up
	35	M	Neg	Pf	Pf	See # 3 (same patient)
	26	M	Neg	Pv	Pv	No malaria tx; presented 2 d later & dx'd w/ Pf → treated for Pf. Follow-up visit 6 d later showed neg smear.
	40	F	Neg	Pv	Pf + Pv + Po	No malaria tx. No follow-up
	15	M	Pv	Pv	Pf + Pv	Tx for Pv only. Follow-up visit 4 d later showed neg smear.
	28	M	Neg	Neg	Pf	No malaria tx; presented 4 d later & dx'd w/ Pf → treated for Pf. Follow-up visit 5 d later showed neg smear.
	13	F	Neg	Neg	Pv	No malaria tx; presented 6 d later & dx'd w/ Pv → treated for Pv. Follow-up visit 3 d later showed neg smear.
	40	F	Neg	Neg	Pv	No malaria tx. Did not deliver baby @ MTC; no follow-up.
	22	F*	Neg	Neg	Pf	No malaria tx; presented 6 d later w/ neg smear.
	73	M	Pv	Pf	Pf	Tx for Pv. Follow-up visit 4 d later showed neg smear.
	36	M	Pv	Pf	Pf	Tx for Pv. Follow-up visit 4 d later showed neg smear.
	27	M	Pv	Pf, g	Pf	Tx for Pv; presented 4 d later & dx'd w/ Pf → treated for Pf. No follow-up.
	11	F	Neg	Neg	Pv	No malaria tx. No planned follow-up.
	26	M	Pv	Pf	Pf	Tx for Pv. No follow-up.
	47	M	Neg	Pf, g	Pf	No malaria tx; presented 3 d later and dx'd w/ Pf → treated for Pf. F/u visit 5 d later showed neg smear.
	38	M	Neg	Neg	Pf	No malaria tx. No planned follow-up.
	38	F	Neg	Neg	Pv	No malaria tx. No planned follow-up.

*Pregnant

Main Quality Indicators

Using PCR as the reference standard and the presence of malaria as the dependent variable (i.e., reflecting each group's ability to discern positive from negative blood smears and not taking species-specific errors into account) sensitivity and specificity of Mae Tao Clinic microscopy were 90.5% (95% CI, 85.8-95.2) and 98.9% (95% CI, 97.4-100.0), respectively; positive predictive value [PPV] and negative predictive value [NPV] were 98.5% (95% CI, 96.5-100.0) and 93.1% (95% CI, 89.6-96.6) respectively. ($p = 0.0001$ for sensitivity and NPV; $p = 0.16$ for specificity and PPV). In comparison, MoPH microscopy exhibited greater sensitivity (93.2%; 95% CI, 89.2-97.2), the same specificity (98.9%; 95% CI, 97.4-100.0), and improved positive and negative predictive values (98.6%; 95% CI, 96.6-100.0 and 95.0%; 95% CI, 91.97-98.03 respectively). ($p = 0.001$ for sensitivity and NPV; $p = 0.16$ for specificity and PPV; Tables 6a and 6b.)

Table 6a: Mae Tao Clinic vs. PCR positive/negative cross-tabulation

		PCR Control		
		+	-	Total
MTC	+	134	2	136
	-	14	189	203
	Total	148	191	339

True positives = 134

True negatives = 189

False positives = 2

False negatives = 14

Sensitivity: 134/(134+ 14) = **90.5%** (95% CI, 85.8-95.2)

Specificity: 189/(189+2) = **98.9%** (95% CI, 97.4-100.0)

Positive Predictive Value: 134/(134 + 2) = **98.5%** (95% CI, 96.5-100.0)

Negative Predictive Value: 189/(189 + 14) = **93.1%** (95% CI, 89.6-96.6)

Table 6b: Ministry of Public Health vs. PCR positive/negative cross-tabulation

		PCR Control		
		+	-	
MoPH	+	138	2	140
	-	10	189	199
		148	191	339

True positives = 138

True negatives = 189

False positives = 2

False negatives = 10

Sensitivity: 138/(138+ 10) = **93.2%** (95% CI, 89.2-97.2)

Specificity: 189/(189+2) = **98.9%** (95% CI, 97.4-100.0)

Positive Predictive Value: 138/(138 + 2) = **98.6%** (95% CI, 96.6-100.0)

Negative Predictive Value: 189/(189 + 10) = **95.0%** (95% CI, 91.97-98.03)

Most real-world quality control studies compare results from the group of microscopists in question with those of expert microscopists. Using MoPH results as the reference standard (instead of PCR), MTC sensitivity was 94.3% (95% CI, 90.5-98.1), specificity 98.0% (95% CI, 96.1-99.9), positive predictive value 97.1% (95% CI, 94.3-99.9), negative predictive value 96.1% (95% CI, 93.4-98.8). ($p = 0.004$ for sensitivity and NPV; $p = 0.04$ for specificity and PPV). See Appendix 2 for calculations and cross-tabulations.

When assessing how well the MoPH microscopists would have performed with only one chance to interpret blood smears, disregarding results that were discordant with MTC interpretations, their sensitivity, specificity, positive predictive value, and negative predictive value were 91.9% (95% CI, 87.6-96.2), 98.4% (95% CI, 96.1-99.9), 97.8 % (95% CI, 95.4-

100.0), and 94.0 % (95% CI, 90.7-97.3) respectively. See Appendix 2 for calculations and cross-tabulations.

Agreement

Sensitivity and specificity measure overall group test performance but do not isolate individual disagreements among cases. To evaluate the degree of agreement among positive and negative results from each of the three groups, unweighted kappa statistics were calculated. Kappa agreement between MTC and PCR results was 0.799 (95% CI 0.723-0.874). Kappa agreement between MoPH and PCR results was 0.889 (95% CI 0.831-0.947). Kappa agreement between MTC and MoPH results was 0.827 (95% CI 0.758-0.896). More detailed calculations and cross-tabulations are presented in Appendix 3.

Slide Quality (Table 7)

Of 339 thick smears, 99 (29.3%) were classified as "good" quality, 34 (10.0%) average quality, and 206 (60.8%) poor quality. For thick smear color, 78 slides were graded as having "good" color (23.0%), 147 "average" color (43.4%), 83 (24.2%) "poor" color, and 32 (9.4%) received no comments. Among the 339 thin smears, 110 (32.4%) were "good" quality, 88 (26.0%) "average" quality, 139 (41.0%) "poor" quality. Two (0.6%) received no comments.

Table 7: Blood Smear quality and color *(n = 339)*

	[illegible]	[illegible]	[illegible]
[illegible]	99 (29.3%)	78 (23.0%)	110 (32.4%)
[illegible]	34 (10.0%)	147 (43.4%)	88 (26.0%)
[illegible]	206 (60.8%)	83 (24.2%)	139 (41.0%)
[illegible]	0	32 (9.4%)	2 (0.6%)

In addition to basic smear evaluation, supplemental, miscellaneous comments were provided for certain slides. Of the 339 slides, 137 (40.4%) were classified "dirty," 48 (14.2%) "very dirty," and 17 (5.0%) contained precipitins from oxidized Giemsa stain.

When correlating slide quality comments with errors made, 27.5% of the 40 MTC misdiagnosed slides had "good" thick smears, 10.0% "average" thick smears, and 62.5% "poor" thick smears. In comparison, 27.4% of the 22 MoPH misdiagnosed slides had "good" thick smears, 9.1% "average" thick smears, and 63.5% "poor" thick smears. ($p = 0.99$). Similarly, 27.5% of MTC misdiagnosed slides had "good" thin smears, 35.0% "average" thin smears, and 37.5% "poor" thin smears, versus MoPH misdiagnosed slides, 18.2% of which had "good" thin smears, 27.3% "average" thin smears, and 54.5% "poor" thin smears. ($p = 0.05$). In terms of additional comments, 45% of MTC misdiagnosed slides were considered "dirty," 12.5% were considered "very dirty," and 2.5% had Giemsa precipitins, compared with MoPH misdiagnosed slides, 36.4% of which were considered "dirty," 18.2% "very dirty," and 9.1% of which had Giemsa precipitins.

Discussions and Recommendations

Discussion

This study showed that the Mae Tao Clinic and the Thai Ministry of Public Health diagnosed malaria with comparable accuracy using microscopy. Even though MTC microscopists made almost twice as many overall errors as MoPH microscopists, most of these errors were species-specific and therefore did not result in any statistically significant difference between the overall main quality indicators for both parties. In particular, the sensitivity and specificity obtained by both groups exceeded World Health Organization Southeast Asia Regional Office [WHO/SEARO] standards for microscopy competency at the regional level (desired sensitivity $\geq$ 90%; desired specificity $\geq$ 95%).[17] Though located in a remote field area, Mae Tao Clinic performed well above the minimal expected standards for a primary diagnostic site (desired sensitivity $\geq$ 80%; desired specificity $\geq$ 85%).[17] Moreover, these results underestimate the performance of MTC and MoPH microscopists, since PCR detects malaria parasitemia at levels five times lower (1-5 parasite/mm^3 blood) than that detectable by the best microscopist[9] (i.e. yielding positive results for subpatent parasitemia). Thus, using PCR as a reference standard will underestimate MTC and MoPH sensitivity and negative predictive value. For this reason, while PCR is an appropriate reference standard for assessing diagnostic test performance, it is not usually used as the reference standard for assessing microscopy quality. Accordingly, it is likely that the MoPH, if compared against a non-PCR gold standard, would meet the WHO/SEARO standards for microscopy at the national level, as it should (desired sensitivity and specificity $\geq$ 95%).[17]

When comparing species-specific and negative/positive agreement among results, the Mae Tao Clinic had good agreement with PCR (kappa = 0.799 (95% CI 0.723-0.874)) and very good agreement with MoPH results (kappa = .827 (95% CI 0.758-0.896)). The MoPH had very good agreement with PCR (kappa = 0.889 (95% CI 0.831-0.947)). MTC microscopists tended to overdiagnose *P. falciparum*, especially in cases of single *P. vivax* infections, to a greater extent than the MoPH. Even though such errors result in unnecessary administration of the more costly artesunate-based regimen and contribute to the problem of resistance against this "last defense" drug, they are less harmful than *P. falciparum* false-negatives, given the potential clinical severity of a *P. falciparum* infection.

Mae Tao Clinic accuracy could have been affected by several factors. At the time of the study, the Mae Tao Clinic laboratory team consisted of 23 Burmese technicians whose microscopy experience ranged from a few months to over 10 years. There is a high level of turnover among staff, since many return to Burma, are resettled to another country, or cannot maintain a long-term position in the laboratory due to personal or political reasons. Consequently, it is difficult to build the expertise level of each team member and ensure a reliable, constant standard of quality. In contrast, the Ministry of Public Health laboratory team consisted of four expert technicians who have each been performing quality control microscopy for the whole of Thailand for over 10 years, following intensive mentored training, strong peer support and interaction, and adhering to a strict protocol outlined by the WHO. Despite the potentially considerable difference in skill level between the two groups, the Mae Tao Clinic performed well.

It is also noteworthy that Mae Tao Clinic staff only had one opportunity to read and diagnose each blood smear, whereas the MoPH had the chance to re-evaluate 38 slides that

were originally discordant with MTC results. Of these, 8 results were changed to a new result, 7 of which agreed with the PCR result. These changes between the initial and final MoPH interpretations are significant, as the MoPH's sensitivity would have been reduced to 91.9% (95% CI, 87.6-96.2) from 93.2% (95% CI, 89.2-97.2), and specificity would have been reduced to 98.4% (95% CI, 96.1-99.9) from 98.9% (95% CI, 97.4-100.0) had they not had the opportunity to re-examine those slides.

The high percentage of misdiagnosed slides with poor quality thick and/or thin smears may indicate that sub-par smear quality impairs the ability of microscopists to diagnose accurately, or it may simply parallel the larger overall proportion of poor quality smears present. However, it does appear that "good" thick or thin smear quality was not a guarantee of an accurate reading. The fact that a larger proportion of MoPH misdiagnosed slides had poor quality thin smears compared to MTC misdiagnosed slides may indicate that that poor thin smear quality was conducive to MoPH errors, whereas MTC errors in reading thin smears may be more attributable to skill level. Despite these ambiguities, improving smearing techniques and stain quality through regular trainings led by a reliable outside agency adhering to WHO protocol would likely improve MTC baseline accuracy in interpreting blood smears. In addition, though collected blood smears were stored appropriately, age and elemental exposure might have caused smear degradation over the four-week sample collection period so that slide quality was diminished by the time they arrived at the MoPH, resulting in a greater number of MoPH errors.

Since over half the misdiagnosed slides for both groups were "dirty" or "very dirty," efforts to reduce residue buildup on slides would probably enhance diagnostic accuracy. Suggested methods for this include washing new slides before use (to eliminate the oily film

that attracts dirt), and using filtered water to wash slides and mix Giemsa stain (to avoid mineral deposition). Though the presence of precipitins from oxidized Giemsa were present on only a few misdiagnosed slides, filtering Giemsa stain and ensuring that ensuring that each batch of Giemsa is used within an hour of mixing would minimize oxidation and prevent precipitins from potentially affecting slide diagnosis.[18]

In this study, MTC and MoPH microsopist performance may have been underestimated, as it was assumed that PCR, the "gold standard" reference test, had 100% accuracy. It is well documented that PCR yields positive results for a longer time after treatment than does microscopy, which in some cases results in false positive diagnosis of an infection that may be clinically insignificant or does not warrant treatment. Srinivasan et al (2000) demonstrated that PCR detected plasmodium DNA in 12 of 13 patients that were apparently aparasitemic by microscopy 2-5 days after treatment.[19] While these findings were thought to represent circulating *plasmodium* DNA from possibly non-viable parasites killed by appropriate drug treatment, Jarra et al (1998) asserts that only viable parasites are detectable by PCR.[20] If Jarra et al are correct, then PCR might still show a positive result for patients with subpatent parasitemia, a partially treated/recrudescent infection, live but damaged parasites that are unable to cause infection, or gametocytemia (presence of sexual forms of the parasite that are transmissible but do not cause clinically significant infection or respond to drug therapy).[21,22, 23] Since the latter two options qualify as a clinically "negative" infection, a positive PCR result for these reasons would be considered a "false positive" result that microscopists would likely diagnose as "negative."

In addition, though PCR protocol was performed carefully and discordant results repeated, sample contamination could have resulted in false-negative or false-positive PCR findings,

which would have mistakenly attributed error to the microscopists. The fact that 49 PCR samples had to undergo amplification a third time because of discordance between first and second interpretations indicates that the potential for error is considerable. In addition to sample contamination and cross-contamination, possible sources of PCR error include skill level of PCR technicians (one who was a relative novice and one who had several years' experience), mechanical error from the PCR or gel electrophoresis equipment that caused reduced sequence amplification or inconclusive visualization of PCR products, and misinterpreting a PCR product on the gel as a false positive or false negative. In addition to the fact that PCR must be performed under near-sterile conditions, the many factors contributing to potential PCR errors emphasize why PCR is unrealistic under field conditions and should not be considered the gold diagnostic standard when ideal laboratory conditions cannot be upheld.

Most patients untreated for *P. falciparum* or *P. vivax* infection at their initial Mae Tao Clinic evaluation (53.8% for untreated *Pf*; 75% for untreated *Pv*) never received proper treatment and were lost to follow-up. However, almost half of patients with initially untreated *P. falciparum* and a quarter of those with initially untreated *P. vivax* returned to the clinic for follow-up and received treatment (albeit sometimes for the wrong species) that ultimately yielded a negative blood smear at a subsequent clinic visit. These observations underscore the importance of being able to access health care services for follow-up visits (which many of the transient MTC patients cannot) and indicate that some *P. falciparum* infections are still susceptible to chloroquine and primaquine, the treatment for *P. vivax*. Despite the favorable outcomes in this subset of patients, however, it is still of paramount importance to diagnose

malaria infections correctly the first time and assume that all *P. falciparum* infections are resistant to chloroquine/primaquine until proven otherwise.

Despite the increasing use of rapid diagnostic testing (RDT), an immunoglobulin-based assay to diagnose malaria antigens, in more remote clinical settings over the years, the WHO still recommends microscopy as the first-line laboratory diagnosis for malaria due to its superior sensitivity and ability to detect multiple malaria species and other conditions .[24] Even though RDTs are preferred in settings where "good microscopy cannot be maintained or is non-existent,"[25] the WHO stresses the importance of extending and strengthening good microscopy facilities "closer to the periphery of health services" in order to more accurately differentiate species and thus dictate drug-specific treatment for *P. falciparum*.[24] Nevertheless, there are those who believe that RDT's, whose results do not depend on subjective interpretation, reliable equipment maintenance, or skill level of the administrator, should be used more widely in a variety of field settings, and that a febrile patient with a negative *P. falciparum* RDT result should be treated empirically for *P. vivax*.

While debates about the best paradigm for malaria diagnosis in the periphery continue, microscopy is still the preferred diagnostic modality along the Thai-Burma border. As one such organization in this region, the Mae Tao Clinic aims to serve as training hub for other clinics across the border in Burma that would like to develop and strengthen their own microscopy facilities. Results from this study demonstrate that the Mae Tao Clinic has been able to implement an adequate malaria microscopy facility in a resource-limited field setting and is thus qualified to take on this responsibility. To further evaluate which diagnostic modalities are most advantageous in the varied clinical settings along the Thai-Burma border, a similar quality control study incorporating blinded comparison of microscopy, RDTs, and

PCR at a field site like the Mae Tao Clinic might be useful. In addition, given the ramifications of missed diagnosis and inappropriate treatment, a standardized, reliable network of monitoring, evaluation, and quality control strategies should be implemented among all health service providers in this highly endemic region.

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หมายเหตุ เพิ่มเติมในการตอบข้อสังเกต และคำแนะนำ จาก ท่านคณะกรรมพิจารณารายงานฉบับสมบรูณ์

งานวิจัยนี้มีวัตถุประสงค์เพื่อที่จะตรวจสอบคุณภาพของการวินิจฉัยโรคมาลาเรียจากฟิล์มโลหิตแบบหนาของเจ้าหน้าที่ของแม่ตาวคลินิก โดยเปรียบเทียบกับผลที่อ่านผลโดยเจ้าหน้าที่ของกรมควบคุมโรค กระทรวงสาธารณสุข ทั้งสองหน่วยงานนี้ใช้ลักษณะทางสัณฐานวิทยาของเชื้อมาลาเรียแต่ละชนิดตามคู่มือขององค์การอนามัยโลกในการจำแนกชนิดของเชื้อ ซึ่งการวินิจฉัยชนิดของเชื้อมาลาเรียด้วยกล้องจุลทรรศน์เป็นวิธีมาตรฐานที่ใช้ในภาคสนาม แต่เนื่องจากการวิธีนี้เป็นวิธีที่ต้องอาศัยความชำนาญของผู้ปฏิบัติงานและมีข้อจำกัดหลายประการ ผู้วิจัยจึงใช้เทคนิค PCR ซึ่งมีความจำเพาะและความไวในการตรวจวิเคราะห์โดยสามารถให้ผลบวกได้แม้มีจำนวนเชื้อเพียงแค่ตัวเดียวและใช้ผลที่ได้จากเทคนิคนี้เป็นมาตรฐานตรวจสอบในห้องปฏิบัติการ ซึ่งสามารถสรุปผลถึงคุณภาพของเจ้าหน้าแม่ตาวคลินิกในการจำแนกเชื้อได้สองระดับ คือ เมื่อเทียบกับเจ้าหน้าที่ของกระทรวงสาธารณสุข และเมื่อเทียบกับผลที่ได้จากเทคนิค PCR