View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Simorgh Research Repository

J Vect Borne Dis 43, June 2006, pp. 53–57

Comparing the results of light microscopy with the results of PCR method in the diagnosis of *Plasmodium vivax*

Ali-Akbar Haghdoost, Shahrzad Mazhari & Kambiz Bahadini

Physiology Research Centre, Kerman University of Medical Sciences, Jomhoori Islami Blvd, Kerman, Iran

Abstract

Background & objectives: Although polymerase chain reaction (PCR) is a new technique in the diagnosis of malaria with very high accuracy; light microscopy is still conventional diagnostic method in Iran. In this study we checked the accuracy of light microscopy using the results of PCR as gold standard in Iran.

Methods: The blood samples were collected from 124 febrile cases in Kahnooj district. The blood slides were read by microscopists, and double checked by experts in provincial referral laboratory. DNA samples were processed by PCR to amplify species-specific sequences of 18s subunit ribosomal ribonucleic acid (18ssrRNA) genes of *Plasmodium vivax* and *P. falciparum.*

Results: The sensitivity and specificity of microscopy in the detection of *Plasmodium* spp infection were 77% (95% CI: 46–94%) and 100% (95% CI: 95–100%), correspondingly. Also, the estimated positive and negative predictive values were 100% (95% CI: 66–100%) and 97% (95% CI: 91–99%), respectively.

Interpretation & conclusion: According to these results, we believe that the accuracy of light microscopy in the diagnosis of malaria in Kahnooj was acceptable. Expert micorscopists in endemic areas of Iran such as Kahnooj and available equipments in one hand and expensive PCR test on the other hand may convince that in current situation we do not have to change the diagnostic method.

Key words Diagnosis – Iran – light microscopy – malaria – PCR

Introduction

Light microscopy has historically been the mainstay of the diagnosis of malaria in Iran. The national health policy of Iran dictates that the clinical diagnosis of malaria disease should depend on visualisation of parasites by light microscopy of Giemsa-stained blood smear in febrile cases. This procedure is cheap and simple, but it requires well-trained personnel.

In Iran, most of the microscopists are technically sound, and there is a quality control programme which supervises the microscopy results. PCR is a useful tool to validate the effectiveness of light microscopy in the detection of malarial parasites. It seems that PCR has greater sensitivity and specificity than light microscopy[1-4], particularly in situations of low-level parasitaemia[5]. Furthermore, it is a more powerful technique to detect mixed infections of

malarial species[6]. However, it is an expensive technique which requires sophisticated laboratory and skillful personnel.

There is only one published report on the accuracy of microscopy results compared to PCR results in Iran[7]. They double checked the microscopy results of 120 fever patients in the Chahbahar district of Sistan and Baluchestan Province in southeastern Iran. They found microscopy had more than 95% specificity in the detection of *P. falciparum* and *P. vivax*. However, this technique missed around 75% of mixed-species malarial infections.

It seems that we need more evidences to make the best decision to approach suspected malaria cases in Iran. Hence, we carried out this study in Kahnooj. Since Kahnooj and Chahbahar, differ in many aspects such as malaria endemicity, socio-economic status and experience of microscopists, we supposed that our results might add valuable information.

The main objective of this study was to compare the results of light microscopy with those of PCR method in the diagnosis of different *Plasmodium* spp. In order to achieve this objective, we estimated the sensitivity, specificity and accuracy of light microscopy results taking PCR results as the gold standard.

Material & Methods

This study was carried out in Kahnooj which is a district of Kerman province in southern Iran with hot dry weather and an area of 32,000 km^2 and around 2,50,000 population.

The blood samples were collected from febrile cases who sought treatment at the rural health centres in three highly endemic villages in the northwest, centre and southeast of Kahnooj with around 10% *P. falciparum* infection. A systematic random sample, based on the days of week, was selected in August and September 2002, when both *P. falciparum* and *P. vivax* cases were at their peak prevalence.

First, informed consent from patients or their guardians was obtained, as was information on the age, sex, duration and symptoms of the disease. Next, finger-prick blood samples were collected: thick blood slides were prepared for microscopical observation, and for comparison; three blood dots were dropped directly on filter mats for the PCR assay.

For the thick blood slide analysis, all the blood slides were air-dried, fixed in methanol and then stained in Giemsa for 15–30 min; a 1 : 10 dilution of Giemsa (pH 7.2) was used. The stain was washed off with tap water. Further, the slides were read by microscopists with the routine methods—oil immersion lens at × 1000 magnification for at least 100 oil immersion fields; also, an expert microscopist in the reference laboratory in the centre of the province rechecked the slides blindly.

For the PCR assay, extraction of parasite deoxyribonucleic acid (DNA) was carried out using a chelex extraction method described by Walsh *et al*[8]. Briefly, the blood samples on filter mats were thawed, and the parasite DNA was extracted by boiling with 20% chelex resin after the samples were left overnight in 1 × PBS/0.5% saponin. DNA samples were processed by PCR to amplify species-specific sequences of 18s subunit ribosomal ribonucleic acid (18ssrRNA) genes of *P. vivax* and *P. falciparum*. All positive samples based on microscopy and ninety of the slide negative samples were also tested by PCR using a 96-well plate. About 15µl DNA was pooled together once into a ‘row’ and once into a ‘column’ from each negative sample. Each pool was cleaned by phenol/chloroform and ethanol, precipitated and re-suspended in 15 µl.

Using Epi-info 6, the data were analysed and the sensitivity, specificity, positive and negative

predictive values (PPV and NPV) and their 95% confidence intervals were computed. These indices are for slide reading, taking PCR as the gold standard.

Results

A total of 124 patients were included in this study, the mean and standard deviation of age were 20.2 and 16.7 years respectively (with minimum and maximum of six months and 77 years), 60 (48.4%) were males and 64 (51.6%) females. All patients had a history of fever in last 48 h prior to seeking treatment and 43% had pain, of which headache was the most common form while 38% had shivering and 6% vomiting (Table 1).

Table 1. Distribution of sex, age and location; and history of symptoms among 124 study subjects

	Frequency	Percentage
Sex		
Male	60	48.4
Female	64	51.6
Location		
Northwest	33	26.6
Centre	67	54
Southeast	24	19.4
Age (year)		
< 10	38	30.6
10–20	43	34.7
21–40	21	16.9
> 40	22	17.7
History symptoms		
Fever	124	100
Shivering	47	38
Pain	53	43
Vomiting	7	6

Table 2. Accuracy of microscopy in the detection of *P. vivax* among fever patients in Kahnooj

PCR / Microscopy	Positive	Negative	Total
Positive	10	0	10
Negative	3	97	100
Total	13	97	110

Sensitivity 77%; Specificity 100%; Positive predictive value 100%; Negative predictive value 97%.

Based on microscopist reports, 10 patients were infected with *P. vivax* and none with *P. falciparum* (Table 2). These results were exactly the same as the referral laboratory reports. The PCR results showed that all of the positive slides for *P. vivax* based on microscopy were also detected as positive by PCR, none of these patients had mixed infection. On the other hand, three of the negative slides for *P. vivax* based on microscopy were detected as positive by PCR (samples in row A and columns 2, 4 and 9 in Fig. 1).

The sensitivity and specificity of microscopy in the detection of *Plasmodium* spp infection were 77% (95% CI: 46–94%) and 100% (95% CI: 95–100%), correspondingly. Also, the estimated positive and negative predictive values were 100% (95% CI: 66–100%) and 97% (95% CI: 91–99%), respectively (Table 2).

Discussion

This study showed that the sensitivity and specificity of microscopy in the detection of *P. vivax* spp infection were 77 and 100%, respectively. Also, the estimated positive and negative predictive values were 100 and 97%, respectively (Table 2). In contrast to the sensitivity and specificity, the predictive values are dependent on the disease prevalence. The blood samples were taken during the peak of malaria transmission season in highly endemic villages. Therefore, it could be expected that the NPV was higher in the whole district—negative blood slide based on light microscopy in the current setting can rule out *Plasmodium* spp infections in febrile cases with at least 97% precision, and positive slides confirm infection with 100% precision. Hence, from a medical point of view, the result of microscopy is a very accurate tool in the diagnosis of malaria.

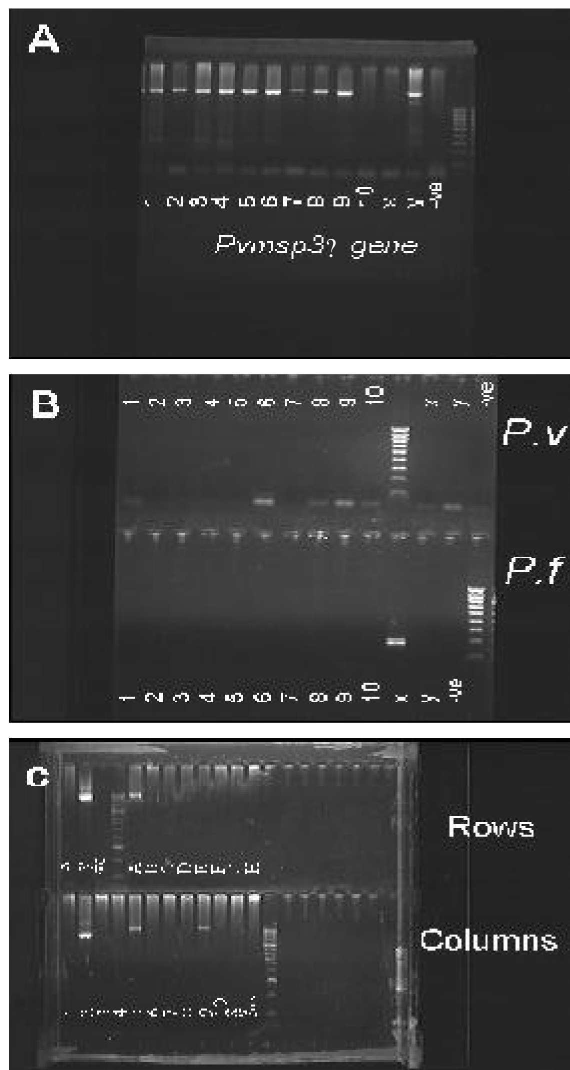

*Fig. 1:*Gel nested PCR products; clinical specimens using species-specific oligonucleotide pairs for A (*PvMSP3?* gene), and B (species-specific amplifications) in *P. vivax* slide positive samples, and C (*PvMSP3*? gene) in pooled slide negative samples, which shows positive PCR in samples in row A and columns 2, 5 and 9. Markers are 100 bp ladders. x: 3D7, y: v97007 (a *P. vivax* gene extracted from Venezuela samples); and –ve: negative control

The three false negative samples were of two females aged 45 and 55 years, respectively, and one male aged 18 years; two of them had a history of fever with vomiting, and the third one had only a history of fever. The blood slides of these three patients were re-read by an expert in the reference laboratory, and 300 fields were assessed per slide. No parasite was detected in any of these slides. Therefore, the discrepant results may either be due to very low levels of parasitaemia and false negative microscopy, or false positive results from the PCR as a result of cross-contamination.

Assuming that the discrepancy between microscopy and PCR is the result of the lower sensitivity of the former method, the reassessment of the slides did not change the primary findings. Therefore, it could be concluded that these slides were from patients with very low levels of parasitaemia. If this is the case, it should be noted that such low-level parasitaemia can rarely be diagnosed by ordinary light microscopic methods. Boisier *et al*[9] showed an association between the level of parasitaemia and fever in an area with seasonal transmission—the signs and symptoms of malaria are not common in very low levels of parasitaemia, which supports others findings even in highly endemic places[10–12]. Therefore, the prese-nce of fever in these three subjects may be due to other febrile diseases which are common during the summer in Kahnooj such as gastroenteritis.

Malaria disease is defined as fever plus positive blood slide with or without other signs or symptoms in endemic areas of Iran. In this project, malaria disease, not infection, is the outcome. Therefore, for the purpose of this study, it can be implied that these three false negative results do not change the accuracy of microscopy results. In other words, since even after very extensive examinations the slides of these three subjects remained negative by microscopy, these patients do not meet the eligible criteria to be classified as malaria patients, therefore, the estimated sensitivity, specificity and predictive values show the accuracy of microscopy in the diagnosis of malaria infection, not the disease.

According to the above explanation, in contrast to the Zakeri *et al*[7] conclusion, we believe that the accuracy of light microscopy in the diagnosis

of malaria in Kahnooj was acceptable. Expert micorscopists in endemic areas of Iran such as Kahnooj and available equipments in one hand and expensive PCR test on the other hand may convince that in current situation we do not have to change the diagnostic method. It seems that the main weak point of light microscopy is in the diagnosis of mixed infections which was highlighted by Zakeri *et al*[7], nonetheless, misclassification of mixed infection does not change the first therapeutic regime in acute phase. Therefore, the impact of missing mixed infections does not have a substantial impact.

Although the sample size might be a point of concern in extrapolating the result of this study, it can be concluded that the light microscopy had satisfactory accuracy in the detection of malaria infection, particularly disease in Kahnooj.

References

1. Singh B, Cox-Singh J, Miller AO, Abdullah MS, Snounou G, Rahman HA. Detection of malaria in Malaysia by nested polymerase chain reaction amplification of dried blood spots on filter papers. *Trans R Soc Trop Med Hyg* 1996; *90*(5): 519–21.

2. Khoo A, Furuta T, Abdullah NR, Bah NA, Kojima S, Wah MJ. Nested polymerase chain reaction for detection of *Plasmodium falciparum* infection in Malaysia. *Trans R Soc Trop Med Hyg* 1996; *90*(1): 40–1.

3. Hanscheid T, Grobusch MP. How useful is PCR in the diagnosis of malaria? *Trends Parasitol* 2002; *18*(9): 395–8.

4. Barker RH Jr, Banchongaksorn T, Courval JM, Suwonkerd W, Rimwungtragoon K, Wirth DF. *Plasmodium falciparum* and *P. vivax:* factors affecting sensitivity and specificity of PCR-based diagnosis of malaria. *Exp Parasitol* 1994; *79*(1): 41–9.

5. Roper C, Elhassan IM, Hviid L, Giha H, Richardson W, Babiker H, Satti GM, Theander TG, Arnot DE. Detection of very low level *Plasmodiumfalciparum* infections using the nested polymerase chain reaction and a reassessment of the epidemiology of unstable malaria in Sudan. *Am J Trop Med Hyg* 1996; *54*(4): 325–31.

6. Urdaneta L, Guevara P, Ramirez JL. Evaluation of DNA recombinant methodologies for the diagnosis of *Plasmodium falciparum* and their comparison with the micro-scopy assay. *Mem Inst Oswaldo Cruz* 1998; *93*(5): 639–46.

7. Zakeri S, Najafabadi S, Zare A, Djadid N. Detection of malaria parasites by nested PCR in southeastern, Iran: Evidence of highly mixed infections in Chahbahar district. *Malar J* 2002; *1*(1): 2.

8. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* 1991; *10*(4): 506–13.

9. Boisier P, Jambou R, Raharimalala L, Roux J. Relationship between parasite density and fever risk in a community exposed to a low level of malaria transmission in Madagascar highlands. *Am J Trop Med Hyg* 2002; *67*(2): 137–40.

10. Lyimo EO, Msuya FH, Rwegoshora RT, Nicholson EA, Mnzava AE, Lines JD, Curtis CF. Trial of pyrethroid impregnated bednets in an area of Tanzania holoendemic for malaria. Part 3. Effects on the prevalence of malaria parasitaemia and fever. *Acta Trop* 1991; *49*(3): 157–63.

11. Lepers JP, Andriamagatiana-Rason MD, Coulanges P. The treatment of *Plasmodium falciparum* malaria in the Malagasy Highland Plateaux: development of fever and parasite density. Clinical aspects. *Arch Inst Pasteur Madagascar* 1989; *56*(1): 155–9.

12. Beadle C, McElroy PD, Oster CN, Beier JC, Oloo AJ, Onyango FK, Chumo DK, Bales JD, Sherwood JA, Hoffman SL. Impact of transmission intensity and age on *Plasmodium falciparum* density and associated fever: implications for malaria vaccine trial design. *J Infect Dis* 1995; *172*(4):1047–54.

Corresponding author: Dr. Kambiz Bahadini, Education Development Centre, Deputy of Education, Kerman University of Medical Sciences, Jomhoori Islami Blvd, Postal Code: 7618747653, Kerman, Iran.
e-mail: kbahaadini@kmu.ac.ir

Received: 29 December 2005 *Accepted in revised form:* 10 May 2006