OPTIMIZING MALARIAL EPIDEMIOLOGICAL STUDIES IN AREAS OF LOW TRANSMISSION

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Abstract. Malaria risk factor studies have traditionally used microscopy readings of blood slides as the measure of malaria infection in humans, although alternatives are available. There is the need for an assessment of how the use of these alternative diagnostic approaches will influence the efficiency and significance of epidemiological studies. In an area of Sri Lanka with known risk factors for malaria, two cross-sectional surveys were done at the start and at the peak of transmission season. Microscopy was compared with enzyme-linked immunosorbent assays (ELISA) and polymerase chain reaction (PCR). The major risk factor in this area was the location of houses relative to confirmed vector breeding sites. At the peak of the transmission season, the results pointed in the same direction, irrespective of the diagnostic method used. However, the importance of distance from the breeding site was not statistically significant when microscopy was used, which can be explained by the lower prevalence of microscopy positivity in comparison to the prevalence of ELISA- and PCR-positivity. This study suggests that in low-transmission areas, such as Sri Lanka, smaller sample sizes can be used for epidemiological research studies using PCR instead of microscopy to estimate parasite prevalence. This efficiency gain has to be weighed against the higher cost and complexity of the PCR. PCR cannot replace microscopy as the standard diagnostic procedure at the field level. ELISA is not directly comparable with microscopy and PCR but it can also be a useful tool in malaria epidemiological studies. This study indicates that cross-sectional surveys are only efficient if they take place during peak transmission season. Cross sectional surveys currently implemented by the Sri Lankan government in response to local malaria outbreaks can form the basis for valid epidemiological studies and be used for the generation of malaria risk maps if samples were also analyzed using PCR.

INTRODUCTION

In spite the fact that alternatives are available most malaria epidemiological studies still use microscopy readings of blood slides as the measure of malaria infection in humans. Similarly, a malaria research project initiated in Sri Lanka in 1994 based its analyses on the results of microscopy. The project identified important risk factors for malaria, and based on this, policy recommendations relevant to the malaria control program in Sri Lanka have been formulated focusing on new approaches for vector larval control in the main breeding sites and the spatial and temporal targeting of residual spraying activities (Konradsen *et al*, 2003). In a case-control study in 7 project villages, the key risk factor identified was house location with people living close to an established vector breeding site having an almost 500% higher risk for malaria than people living further away (van der Hoek *et al,* 2003). Likewise, an earlier prospective study in just one of the 7 villages had also pointed to the same risk factor, although in that study it did not reach statistical significance (van der Hoek *et al*, 1998). Prior to the analytic studies, two baseline cross-sectional parasite surveys were conducted in one of the 7 study villages in 1994. Malaria was assessed with microscopy but blood

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samples from these two cross-sectional surveys were also conserved for later analysis. This provided the opportunity to compare different diagnostic approaches when assessing malaria infection in risk factor studies. To this extent, the results of the enzyme-linked immunosorbent assays (ELISA) and polymerase chain reaction (PCR) tests were compared with microscopy as the outcome measure for the major risk factor for malaria. Likewise, the study aimed at discussing the implications of the three different diagnostic approaches for the sample size, and therefore, efficiency of a cross-sectional epidemiological study.

MATERIALS AND METHODS

Study area

The study was done in a traditional rice-farming village, which had 294 inhabitants at the start of the study (Mahameegaswewa, North-Central Province of Sri Lanka). Details of landscape and meteorological information for the village have been described elsewhere (Amerasinghe *et al*, 1997). A small stream close to the village had previously been identified as the breeding site for the mosquito vector for malaria in this area (Amerasinghe *et al*, 1997).

Sample collection

Two cross-sectional surveys were carried out in the village in 1994. Survey 1 was carried out in September, at the beginning of the malaria transmission season, and Survey 2 in December, during the peak transmission season. During each survey the village residents had blood taken for malaria microscopy and two capillary tubes of blood were obtained for serology after informed consent. Capillary blood was spun for serum collection and cellular components and sera were stored separately at -20ºC until use. ELISA and PCR were performed on the serum and cellular components, respectively.

Microscopy

Blood films (thick and thin smears) were prepared from finger pricks and stained with Giemsa stain, pH 7.4, in the field. All persons with a positive blood slide were treated with chloroquine and primaquine according to the national guidelines of the Anti Malaria Campaign of Sri Lanka. An experienced research laboratory technician examined the slides in a field laboratory, reading 250 microscopy fields under oil immersion on thick smears with at 600 times magnification, followed by a second reading of the slide in the field by a senior microscopist of the Anti Malaria Campaign who was blinded to the previous result and made use of a different microscope. The field microscopy examination was confirmed at the University of Peradeniya, Sri Lanka, within four days of the initial examination.

ELISA

ELISAs were carried out for the detection of antibodies to *P. vivax*-210, *P. vivax*-247 and *P. falciparum* circum-sporozoite proteins (CSP). Synthetic or recombinant peptides of *P. vivax* (Pv 210), *P. vivax polymorph* (Pv 247) and *P. falciparum* were used in the assay with minor modifications, details of which are described elsewhere (Wirtz *et al*, 1989, 1992).

The CSP antibody prevalence in the study population was compared with the sera of 59 individuals between 18 and 40 years of age living in the non-malarious highlands of Sri Lanka. The scatter plots for the optical density (OD) difference (against its serum control) showed that the cut-off values were different for the different species specific ELISAs. The outlier samples were included only after questioning as to whether the persons had been travelling or been to a malarious region. Cut-off values were calculated as mean OD for non-malarious population plus 3 standard deviations, (Pf=0.099; Pv-210=0.086; Pv-247=0.121). Pv-210 and Pv-247 positives were combined for analysis, as the microscopic and PCR methods do not distinguish between the vivax CSP polymorphs. The ELISA on the samples was conducted late in 1999.

DNA extraction and PCR genotype analysis

Unsealed capillary tubes containing approximately 20 μl of packed red blood cells from donors were placed into 1.5 ml eppendorf tubes and incubated over night on a shaking table in 250 μl proteinase K solution at 37ºC (1 mM EDTA, 15 mM TRIS, 150 mM NaCl, 1% SDS, 100 μg/ml proteinase K) (Roche, Hvidovre, Denmark, cat.

744 723). The samples were then extracted by phenol/chloroform and ethanol precipitation as described by Sambrook *et al* (1989).

The diagnostic species PCR was based on a nested PCR amplifying species specific fragments of the *Plasmodium* small subunit ribosomal RNA (ssrRNA) genes using primers described by Singh *et al* (1999). The PCR was however modified into a single round semi-quantitative real-time PCR using a Rotorgene thermal cycle system (Corbett Research). Reactions were performed in 20 μl volumes using QuantiTech SYBR Green PCR master mix including 1.5 mM $MgCl_2$ according to the manufacturers instructions (Qiagen, Denmark), 0.25 μM of each primer pair (rFAL1/rFAL2 or rVIV1/rVIV2 for *P. falciparum* and *P.vivax*, respectively) and 2.5 mM $MgCl_2$. One microliter of extracted DNA was added to the reaction mix. The PCR conditions were 95ºC for 15 minutes, followed by 45 cycles at 95ºC for 30 seconds, 58ºC for 40 seconds and 72ºC for 40 seconds and final extension at 58ºC for 2 minutes and 72ºC for 5 minutes. Data acquisition was done at the end of the elongation of each cycle. The specificity of the amplification was ascertained by melting-curve analysis of each PCR product and often confirmed by gel electrophoresis. The threshold of detection of positive *P. falciparum* or *P.vivax* samples was based on melting-curve analyses comparing sample curve patterns to *P. falciparum* or *P. vivax* standards and a 20% detection threshold based on non template controls. The semi-quantification of the *P. falciparum* positive samples was performed using ROTORGENE software, version 4.6 by comparing obtained Cycle threshold (Ct) values of samples with Ct values from a *P. falciparum* 3D7 isolate used as a standard with known parasitemia. The sensitivity of the PCR was defined for *P.falciparum* by testing a 10-fold dilution series of the 3D7 isolate. The *P. falciparum* quantitative PCR was able to detect ≥1 parasite/μl (data not shown). The PCR sensitivity estimated for *P. falciparum* was also used to define the sensitivity for *P. vivax*. This is likely to be a conservative estimate for the *P. vivax* quantitative PCR since all forms of the parasite are found in the peripheral blood and therefore have a greater number of copies of DNA compared with *P. falciparum.* The PCR of the samples was analysed during early 2003.

Data analysis

The data were analyzed for those village residents for whom the results of microscopy, ELISA, and PCR were available for one or both surveys. The location of the houses in the village was determined with global positioning system receivers. For each house, the shortest distance to the local stream, which was the established vector breeding site, was calculated using Arcinfo geographical information system software (van der Hoek, 1998). Statistical analyses were done with Epi Info 2000 version 1.1 and SPSS version 10.

Table 1
Percentage positives of two cross-sectional surveys in Mahameegaswewa, Sri Lanka, using three different diagnostic methods.

Diagnostic approach	*Plasmodium* species	Survey 1 % positive (n=223)	Survey 2 % positive (n=195)
Microscopy	*P. falciparum* only	2.2	6.7
	P. vivax only	1.8	0
	Mixed	0	1.5
PCR	*P. falciparum* only	10.3	22.0
	P. vivax only	9.9	9.2
	Mixed	0.9	5.6
ELISA	*P. falciparum* only	5.9	8.8
	P. vivax only	5.9	8.3
	Mixed	0	3.6

Survey 1 = early transmission season and Survey 2 = peak transmission season.

Table 2
Percentage positives of two cross-sectional surveys in relation to the distance of the house to the established vector-breeding site in Mahameegaswewa, Sri Lanka.

Survey 1 Distance (meters)	N	Microscopy % positive	PCR % positive	ELISA % positive
<500	145	5.5	21.4	11.7
≥500	78	1.3	20.8	11.8
χ^2		p=0.129	p=0.917	p=0.979
Survey 2 Distance (meters)				
<500	123	12.2	47.2	28.1
≥500	72	5.8	17.4	7.4
χ^2		p=0.154	p<0.001	p=0.001

Survey 1 = early transmission and Survey 2 = peak transmission

RESULTS

Data were available for 223 people in survey 1 and 195 in survey 2. There was a marked increase in the prevalence of malaria parasites/antimalaria antibodies from the first to the second cross-sectional survey, except for *P. vivax* when using microscopy (Table 1). The number of mixed infections increased markedly from the first to the second survey. On the first cross-sectional survey, early in the transmission season, there was no statistically significant difference in the prevalence of malaria parasites (*P. falciparum* and *P. vivax* combined) or antimalaria antibodies, between people living within 500 meters of the local stream and people living further away (Table 2). However, at the second cross-sectional survey, during the peak of the transmission season, living close to the stream came out as a risk factor. Microscopy, PCR, and ELISA showed the same pattern but because of a lower prevalence, the difference was not statistically significant when microscopy was used.

Based on the prevalence figures in the second survey, the sample sizes needed to detect a difference for the risk factor of interest, with a significance level of 5% and a power of 80%, which was 776 using microscopy, 141 using ELISA, and 97 using the PCR.

The sensitivity of the PCR, using microscopy as the gold standard, was 89% and the specificity was 76% (Table 3).

Table 3
Comparison of PCR and microscopy in two cross-sectional parasite surveys in Sri Lanka.

	Microscopy +	Microscopy -	Total
PCR +	25	94	119
PCR -	3	296	299
Total	28	390	418

A total of 62 samples were found to be *P. falciparum* positive by PCR but negative by microscopy (data not shown). When comparing the mean Cycle threshold (Ct) values, as a semi-quantitative measure of parasitemia for these samples, with the 17 samples that were *P. falciparum* PCR positive and microscopy positive, a significantly higher Ct-value was found for the PCR positive but microscopy negative samples ($p < 0.05$). This indicates a significantly lower parasitemia in the PCR positive but microscopy negative samples compared with the PCR positive and microscopy positive samples.

DISCUSSION

Living close to vector breeding sites is a strong and established risk factor for malaria in Sri Lanka (van der Hoek *et al,* 2003) and elsewhere (Foley *et al,* 2003). In the present cross-sectional study at the peak of the transmission season, the results pointed in the same direc-

tion, irrespective of the diagnostic method used. The importance of the distance to the breeding sites was not statistically significant when microscopy was used, which can be explained by the lower prevalence with microscopy compared to the prevalence of ELISA- and PCR-positivity.

In the cross-sectional survey at the beginning of the transmission season, the location of houses relative to vector breeding sites did not come out as a significant risk factor with any of the diagnostic approaches. This is a reflection of the much lower prevalence at that time. It suggests that any risk factor study in a low transmission season in a country with unstable malaria would be highly inefficient.

The findings of this study suggests that in low-transmission areas, such as Sri Lanka, smaller sample sizes can be used in epidemiological research studies by using PCR instead of microscopy to estimate parasite prevalence. Although ELISA measures anti-parasite antibodies and is therefore not directly comparable with microscopy and PCR, the data confirm that it can be a useful and efficient tool in malaria transmission studies as has previously been proposed by Del Giudice *et al* (1990). The efficiency gained by PCR has to be weighed against the higher cost and complexity. Although well trained and experienced technicians are needed to ensure good results from microscopy the capacities, resources and protocols are more demanding in the case of PCR analysis and to a lesser extent for ELISA. This may limit the relevance of the more advanced diagnostic approaches for smaller research projects. Furthermore, it is important to realize that in field-based epidemiological studies it would be very difficult to replace microscopy with PCR or ELISA completely, since microscopy provides a quick malaria diagnosis. It is unlikely that a research activity would have achieved a high participation from the community if people had to wait a long time for a result of testing. However, the collection of an additional drop of blood on a filter paper for later PCR analysis is likely to be worthwhile for epidemiological studies in areas with a low case load.

It is assumed that the half-life of CSP antibodies detected by ELISA is sufficiently short to indicate a present or recent infection with malaria (Webster *et al,* 1987). However, the very sharp increase in the prevalence in antibodies against *P. vivax* found in this study from the first to the second survey might still be a reflection of the fact that the ELISA not only identifies the present infection but captures antibodies generated in response to infections that have occurred between the two surveys.

In a review of the different techniques for the diagnosis of malaria, Makler *et al* (1998) discussed the advantages and disadvantages of the various approaches. The sensitivity and specificity of PCR-based methods, using examination of blood smears as the “gold standard”, was above 90%. In this study the sensitivity and specificity did not quite reach this level was still comparable to previous studies.

Samples that were PCR positive, but microscopy negative, had very low levels of parasite DNA. Therefore, when comparing the results based upon PCR and microscopy analysis the relevance of the very low level of parasitemia detected on PCR will have to be taken into account. Although it is now assumed that malaria parasites detected in the blood through PCR are actually viable parasites (Jarra and Snounou, 1998) it does not necessarily mean that the identified infection would actually result in clinical disease. The same could be said for malaria parasites detected on microscopy. It is likely that the capability of PCR to detect extremely low levels of parasitemia may make it harder to relate the identification of parasites with clinical outcomes. It is difficult to explain the three microscopy positive but PCR negative slides. However, one possible explanation relates to possible PCR inhibitors in the blood, but this was not analyzed in any detail.

The malaria control program in Sri Lanka, as implemented by the Anti Malaria Campaign, involves parasite surveys in areas that are affected by outbreaks of malaria. With PCR it is possible to detect very low levels of parasitemia during these surveys and in this way the possible parasite reservoir in the population could be reduced. Likewise, the findings of this study indicates that the samples collected during the routine cross-sectional surveys could form the

basis for efficient epidemiological studies, especially if the samples were tested using PCR.

In conclusion, this study indicates that in low-transmission areas, such as Sri Lanka, smaller sample sizes can be used in epidemiological research studies by using PCR instead of microscopy to estimate parasite prevalence. This provides an opportunity for the use of blood samples collected by the Sri Lanka Ministry of Health during localized malaria outbreaks as the basis for risk factor studies. However, a wider feasibility study assessing the cost implications and the capacity needs must be carried out before a final recommendation is made for the inclusion of PCR analysis of routinely collected samples for research purposes.

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