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EPIDEMIOLOGIC TOOLS FOR MALARIA SURVEILLANCE IN AN URBAN SETTING OF LOW ENDEMICITY ALONG THE COLOMBIAN PACIFIC COAST

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Abstract. An evaluation of 3 different methods for malaria diagnosis was carried out in an urban area of low endemicity on the Pacific coast of Colombia. Samples were collected from 833 symptomatic patients at a malaria clinic and examined by the polymerase chain reaction (PCR), quantitative buffy coat (QBC™; Becton Dickinson, Franklin Lakes, NJ) method, and the traditional thick blood smear. The prevalence of *Plasmodium falciparum* malaria was 5.88% by thick blood smear, 7.34% by the QBC method, and 21.87% by PCR. The agreement between microscopists was 99.5%. The agreement between the QBC method and thick blood smear was 96.13% (n = 745). Samples positive by PCR but negative by thick blood smear or conversely negative by PCR and positive by thick blood smear were usually of low-level parasitemias. All 3 methods showed agreement in 76.3% of the samples. Sixty-nine (18.8%) samples were positive by PCR but negative by the other 2 methods. Ten samples were positive by both the QBC method and thick blood smear but negative by PCR; most of them had low-level parasitemias. The use of malaria diagnostic methods for epidemiologic surveillance is discussed.

Malaria is endemic on the Pacific coast of Colombia. The population of the 4 coastal states is nearly one million (2.5% of the national population), and accounts for 12.5% of the total malaria cases in the country. There are villages with an annual parasite index as high as 270 per 1,000, and in Buenaventura, the largest town on the Pacific Coast, the corresponding figure was 26.7 per 1,000 inhabitants in 1993 (Rojas O, Carrasquilla G, 1994, unpublished data). In this port city, 30–40% of the total cases reported between 1991 and 1993 came from the urban area. Seventy percent of the cases were concentrated in a geographic area where only 23% of the urban population lives.[1]

It is widely accepted that in most places, the number of malaria cases is underestimated. However, when improvements are made to the data collection system, the reported occurrence of malaria increases significantly. This phenomenon was well documented in the Colombian state of Antioquia, where the reported number of cases has increased 2-fold since 1991, mostly due to improvements to the malaria surveillance program by primary health workers.[2] Diagnostic techniques for large-scale epidemiologic studies are required for endemic areas to establish prevalence, study transmission, and to evaluate control measures.

The routine identification of infection with *Plasmodium* species currently depends upon microscopic examination of Giemsa-stained slides. However, microscopy is time-consuming, limited in sensitivity, and the accuracy of the diagnosis depends greatly on the expertise and diligence of the microscopist.[3] Microscopic analysis is appropriate for routine clinical malaria diagnosis; however, the technique is impractical for large-scale surveillance and field research projects.

The Quantitative Buffy Coat (QBC™; Becton Dickinson, Franklin Lakes, NJ) technique is a rapid method extensively used in clinical as well as in field epidemiologic studies for malaria detection.[4–7] The *Plasmodium* species are identified based on parasite morphology, after staining with acridine orange, and visualization under a fluorescent microscope. The sensitivity and specificity of the technique varies between investigators.

The polymerase chain reaction (PCR) technique permits the analysis of numerous samples concurrently, and has high sensitivity and specificity. Therefore, it can be used to study post-treatment parasite clearance as well as be used to distinguish between reinfection, recrudescence, or resistance.[8,9] The PCR also permits tracking parasite strains throughout a population. Molecular techniques such as the PCR facilitate large-scale epidemiologic studies and surveillance, resulting in better management of the disease by public health officials. This project provides the basis to improve malaria management and control, and for establishing PCR diagnosis for ongoing and future studies on the Pacific coast of Colombia.

The objective of the present report was to evaluate 3 malaria diagnostic methods: the thick blood smear, the QBC method, and a PCR-based technique, with a long-term goal of implementing molecular diagnostics in Buenaventura for epidemiologic surveys.

MATERIALS AND METHODS

Study population and study design. This study was carried out in Buenaventura, a city of 260,000 inhabitants, which is the main port on the Pacific coast of Colombia. Eighty percent of the population lives in the urban area.

The local climate favors the presence of malaria throughout the year; 8,000 mm of rain falls annually, and the average temperature is 26°C (78.8°F). *Anopheles albimanus* is the most common vector in the urban area, while *A. nuneztovari* and *A. (k) neivai* are common in rural zones.[10] A recent *in vivo* study showed that 52% of the malaria cases were resistant to chloroquine (Barrera L, Carrasquilla G, Mendez F, unpublished data).

Patients with self-described malaria symptoms from both urban and rural areas report to the malaria clinic or the 200-bed district hospital in Buenaventura for diagnosis and treatment. The standard diagnostic method for malaria in Buenaventura and elsewhere in Colombia is microscopic examination of Giemsa-stained thick blood smears. Between

July and November 1994, 833 samples were obtained from those attending the malaria clinic in Buenaventura. Informed consent was obtained from all adult participants and from parents or legal guardians of minors. The study was reviewed and approved by the Fundacion para la Educación Superior Ethical Review Board. In an interview with trained laboratory technicians, the patients were asked about malaria symptoms and demographic information. Gender, place of residence, previous malaria episodes, self-treatment with antimalarials, and malarial symptoms were recorded. Those patients who were positive for malaria after diagnosis by the clinic's microscopist were provided with the Ministry of Health's standard treatment (chloroquine, 25 mg/kg in 3 doses; sulfadoxine-pyrimethamine, 25 mg/kg and 1.25 mg/kg, respectively; primaquine, 0.6–0.9 mg/kg in 1 dose).

Sample collection and processing. Samples were obtained at the Malaria Clinic in Buenaventura, Colombia from patients with self-assessed symptoms of malaria. A total of 833 samples was collected in 1994 by digital puncture and used in this study.

Citrate-treated capillary tubes were used to collect blood samples (20 μl, 2 two tubes per patient) for the PCR diagnosis.[8] The blood was transferred into alternate wells of a microtitration plate containing 200 μl of saponin lysis solution (0.2% NaCl, 0.01 5% saponin, 2 mM EDTA). The lysed samples were filtered through no. 903 paper (Schleicher & Schuell, Keene, NH) using a minifold vacuum filtration apparatus, air-dried, and stored at room temperature until processed for the PCR at the University of Puerto Rico.

Microscopy. Thick blood smears (n = 833) were prepared at the time of blood collection from each patient. The slides were air-dried and stained with Giemsa, and subsequently examined by 2 expert microscopists in a double-blind fashion at the Malaria Clinic and at the Instituto de Salud del Pacifico (INSALPA) (Buenaventura, Colombia). One hundred fields were examined before reporting a sample as negative. Parasitemia was calculated by considering 100 fields as 0.250 μl,[11] and multiplying the reported number of parasites by 4 to calculate number of parasites/μl.

Quantitative buffy coat method. Fifty-five microliters of patient's blood were transferred to a QBC tube and mixed.[5] The tubes containing samples were centrifuged and analyzed with a fluorescent microscope by a microscopist within 2–3 days after collection at INSALPA. A second microscopist attempted to read 726 of the 833 sample tubes 4–8 days after blood collection at the Universidad del Valle in Cali. This second set of samples was found to be degraded, and the data could not be used.

Primer and target DNA titration. A set of *P. falciparum*-specific oligonucleotide primers described by Tirasophon and others,[12] which generate a 206-basepair PCR product, were used in the study (5′-CGCTACATATGCTAGTTGCCAGAC-3′ and 5′-CGTGTACCAATACATCCTACCAAC-3′). At the beginning of the study, both primers and target DNA were titrated using 2-fold serial dilutions. For PCR primers, a range of 1.25–50 pmoles were used to amplify 20 ng of target DNA/reaction. Similarly, 2-fold dilutions of *P. falciparum* genomic DNA (5–100 ng/reaction) were titrated using 5 pmoles of primer. Based upon these preliminary results, we used 5 pmoles of primers in all subsequent experiments and 20 ng of genomic DNA in the positive control reactions.[13]

Amplification of DNA by the PCR. Patient samples (n = 448) were brought to the University of Puerto Rico in 1997 for processing. The PCRs were carried out as previously described[12,14] with some modifications. Briefly, a 3-mm circle of the sample was excised from the filter using a disposable biopsy punch (Acuderm, Inc., Fort Lauderdale, FL) and transferred to 0.25-ml vials containing 50 μl of PCR mixture (70 mM Tris, 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100, 50 pg/ml of bovine serum albumin, 0.2 mM deoxynucleotides, 5 pmol of oligonucleotide primers), and 1.25 units of *Taq* polymerase (Amplitaq®; Perkin-Elmer Cetus, Norwalk, CT). The samples were amplified in a thermocycler (GeneAmp PCR System 2400; Perkin-Elmer Cetus) for 30 cycles (1 min at 94°C, 3 min at 37°C, and 5 min at 72°C) and visualized by staining with ethidium bromide after electrophoresis on 2.2% agarose gels. Each set of reactions included the following controls: 1) a positive control containing 20 ng of *P. falciparum* genomic DNA, 2) a negative control containing no target DNA, and 3) a negative control containing no primers.

Hybridization of DNA. The PCR products from some patient samples (n = 188) as well as titration reactions were analyzed by Southern or dot-blot hybridization. Briefly, one-tenth (5 μl) of the PCR product was transferred to nylon membranes (Nytran®; ICN; Costa Mesa, CA) in a dot-blot apparatus (Bio-Rad, Hercules, CA) using vacuum filtration, following the manufacturer's recommendations. For Southern blot analysis, 5 μl of each PCR product were electrophoretically resolved on a 2.2% agarose gel and electroblotted onto Genescreen Plus® hybridization membranes (Dupont, Wilmington, DE). The membrane-bound PCR products were denatured and neutralized followed by hybridization to a ^{32}P random primer labeled *P. falciparum* probe.[15] The primer set previously described was used to amplified the *P. falciparum* DNA fragment that served as probe. The hybridization membranes were washed and exposed to x-ray film (X-Omat®; Eastman Kodak, Rochester, NY).[8]

Statistical analysis. A database was prepared in DBASE IV[16] using the information from the interview and the results of the blood examination. Agreement between observers for the different tests was derived in 2 × 2 tables. Differences between 2 means were evaluated by *t*-tests and by chi-square tests for differences in proportions. Fisher's exact test was used when the numbers in the cells of the tables were very scarce. When comparing several means, one-way analysis of variance was used. The 95% confidence intervals were calculated for parameters.[17] Sensitivity, specificity, and predictive values were calculated based on the PCR as the gold standard to compare with the QBC method and the thick blood smear.[17] A comparison between the last 2 techniques was made. The data were analyzed using STATA® software.[18]

RESULTS

A total of 833 patient samples were collected between July and November 1994 at the malaria clinic in Buenaventura. All samples were analyzed by thick blood smears,

TABLE 1
Prevalence (95% confidence intervals) of malaria infection by 3 different diagnostic methods in Buenaventura, Colombia*

Parasite	Thick blood smear	QBC	PCR
P. falciparum	5.88 (4.38–7.70)	7.34 (5.58–9.45)	21.87 (18.13–25.99)
P. vivax	5.40 (3.97–7.16)	7.08 (5.34–9.15)	
P. malariae	0.36 (0.07–1.04)		
Negative	88.36 (85.98–90.46)	85.58 (82.86–88.02)	78.13 (74.01–81.87)
Total (n)	833	749	448

* QBC = quantitative buffy coat; PCR = polymerase chain reaction; *P.* = *Plasmodium*.

while 749 were examined by the QBC method and 448 by the PCR.

Prevalence of malaria. Comparison of the QBC method and thick blood smears to assess *P. falciparum* and *P. vivax* prevalence exhibited no statistically significant differences. However, PCR diagnosis showed a 3-fold increase in the prevalence of *P. falciparum* compared with the QBC method and a 3.7-fold increase when compared with thick blood smear examination (Table 1).

Parasitemias of different parasite species. There was no difference in the average level of parasitemia in the population infected with *P. falciparum* and those infected with *P. vivax*. The mean *P. falciparum* parasitemia was 1,054.78 parasites/μl (95% confidence interval [CI] = 712.48, 1,397.07) while patients infected with *P. vivax* had a mean of 888.98 parasites/μl (95% CI = 563.36, 1,214.59). The patients with *P. malariae* had significantly lower levels of parasitemia (mean = 236.0 parasites/μl, 95% CI = 378.86, 850.86). However, it must be taken into account that there were only 3 cases yielding very wide CIs.

Agreement between microscopists. Thick blood smears were scored independently by 2 experienced microscopists. The agreement (including parasite species identification) between the two microscopists was 99.5% (829 of 833 samples). Three patients were diagnosed positive by 1 microscopist but negative by the other. Two of these 3 samples were positive for *P. falciparum* infections with parasitemias of 1,364 parasites/μl and 20 parasites/μl, respectively. The former was negative by the QBC method (PCR not done), and the latter was diagnosed as a *P. falciparum* infection by the QBC method (PCR not done). The third of these samples (408 parasites/μl) was identified as *P. vivax*. This sample was also positive for *P. vivax* by the QBC method, and positive for *P. falciparum* by the PCR, suggesting an undetected mixed infection. A fourth discrepancy between the microscopists (1,776 parasites/μl) was diagnosed as a *P. vivax* infection by 1 microscopist and *P. falciparum* by the other. This fourth sample was scored as *P. falciparum* by the QBC method, but was negative for *P. falciparum* by the PCR.

TABLE 2
Comparison of malaria diagnosis to the quantitative buffy coat (QBC) and thick blood smear in Buenaventura, Colombia*

QBC	Thick blood smear			Negative	Total
	P. falciparum	*P. vivax*	*P. malariae*		
P. falciparum	41	1	0	13	55
P. vivax	0	42	3	8	53
Negative	4	0	0	637	641
Total	45	43	3	658	749

* *P.* = *Plasmodium*.

Quantititative buffy coat method. Seven hundred forty-nine (89.9%) of the thick blood smears were also examined by the QBC method. There were 84 samples that could not be evaluated by the QBC method because either the floating coat or the sample was lost. We had originally planned to read QBC samples in duplicate, first in the field, and then again in the laboratory 3–5 days later. However, the second set of samples was found to be degraded, and could not be used in this study.

Comparison of methods. *Comparison of the QBC method and the thick blood smear.* Table 2 compares thick blood smear examination and the QBC method, including malaria species identification. The agreement between the 2 tests was 96.13%. Of the 749 samples, 730 had the same diagnosis by both methods. The three *P. malariae* infections were diagnosed as *P. vivax* by the QBC method. Four (0.6%) of the 641 QBC-negative samples were positive by thick blood smear. Three of the 4 samples positive for *P. falciparum* by thick blood smear and negative by the QBC method had very low parasitemias (4–8 parasites/μl). Two of these 4 samples were tested by a *P. falciparum*-specific PCR; both of them were positive. One sample, which was diagnosed as *P. vivax* by a thick blood smear and *P. falciparum* by the QBC method, had a parasitemia of 1,776 parasites/μl and was negative by the *P. falciparum*-specific PCR, so the thick blood smear diagnosis of *P. vivax* was probably correct.

Comparison of the PCR and the thick blood smear. To validate the *P. falciparum*-specific PCR method, it was important to assess whether the population of patients whose blood samples were tested by the PCR differed from the population whose samples were not analyzed by this procedure. Comparison of demographic (age, gender, place of residency) and malaria (symptoms, parasitemia, prevalence of *P. falciparum)* variables showed that the subset of blood samples diagnosed by the PCR did not differ that of the entire studied population.

The comparison of diagnosis by the PCR and thick blood smear (448 samples) is shown in Table 3. Among the thick blood smear–negative samples, 78 (19.6%) were positive by the PCR. Of these 78 PCR-positive samples, 74 gave a weak signal by hybridization, reflecting a low level of parasitemia. On the other hand, 12 of 29 blood smear-positive but PCR-negative samples were *P. falciparum* infections. Seven had fewer than 400 parasites/μl, but 5 had higher numbers (412–3,260/μl).

Comparison of the QBC method and the PCR. As in the comparison between blood smear examination and the PCR, there was a large number of samples positive by the PCR but negative by the QBC method (Table 3). Seventy one (77.2%) were positive by the PCR, of which 67 gave a weak

TABLE 3

Comparison between the polymerase chain reaction (PCR) with the thick blood smear and the quantitative buffy coat (QBC) method in Buenaventura, Colombia*

PCR	Positive	Negative	Total
Thick blood smear			
P. falciparum	10	12	22
P. vivax	10	17	27
Negative	78	321	399
Total	98	350	448
QBC			
P. falciparum	9	16	25
P. vivax	12	16	28
Negative	71	272	343
Total	92	304	396

* *P.* = *Plasmodium*.

TABLE 4

Sensitivity and specificity of 3 methods for *Plasmodium falciparum* malaria diagnosis in Buenaventura, Colombia*

	Thick blood smear vs. QBC	PCR vs thick blood smear	PCR vs QBC
Sensitivity (%)	91.11	10.20	9.78
Specificity (%)	98.02	96.57	94.74

* QBC = quantitative buffy coat; PCR = polymerase chain reaction.

signal. The QBC method exhibited a low sensitivity of 9.8% compared with the PCR, with a specificity of 94.7%, which is lower than the thick blood smear due to the large number of samples read as positive by the QBC method. Of these 16 samples, 10 were also positive by the thick blood smear as stated above. There were 5 samples positive by the QBC method but negative by the other 2 methods, suggesting a false-positive diagnosis, and 1 sample was negative by the PCR but *P. vivax*-positive by the thick blood smear.

Agreement between the 3 methods of diagnosis. Table 4 shows a comparison of sensitivity and specificity between the 3 methods. Both the QBC method and the thick blood smear have low sensitivity when compared to a PCR gold standard, but each has a specificity of approximately 95%. The sensitivity of the thick blood smear compared with the QBC method for the diagnosis of *P. falciparum* malaria was 87.2% and the specificity was 97.7%.

Table 5 shows the agreement in diagnosis for the 367 patients from whose blood samples were tested by all 3 methods. All 3 methods agreed in 76.3% of the samples (272 [74.1%] negative and 8 [2.2%] positive). Sixty-nine (18.8%) samples were positive by the PCR but negative by the other 2 methods. However, 10 samples were positive by both the QBC method and the thick blood smear but negative by the PCR. Six of them had less than 1,000 parasites/μl. All had parasitemias less than 3,300 parasites/μl.

DISCUSSION

Conventional microscopic examination of thick blood smears, the QBC method, and the PCR represent different methods for detecting malaria parasites. These techniques differ with respect to ease of use, limits of detection, and ability to identify the target species. Furthermore, genetic techniques such as the PCR offer the opportunity to do more than simply diagnose the presence of malaria infection, since these techniques can also be used to determine levels of drug resistance.[19–21] To evaluate the sensitivity and specificity of the 3 methods, a cross-sectional study was conducted at a malaria clinic in Buenaventura, Colombia.

The prevalence of falciparum malaria in the study population was 5.9% by thick smear, but 21.9% by the PCR. These levels are comparable with those previously reported in and around Buenaventura, an area of low endemicity. For example, Gautret and others[22] reported a prevalence of 8.5% in a village near Buenaventura while Terrientes and others[23] found a relatively high prevalence of 33.1% at Punta Soldado on the Colombia Pacific coast. The mean parasitemia in our study population was also relatively low, with an average of 1,054.78 parasites/ml in *P. falciparum*-infected individuals and 888.98 parasites/ml in *P. vivax* infections. This may be due to the relatively low frequency of infection. The low parasitemia may reduce sensitivity and complicates detection by microscopic examination.[24,25] We found a prevalence ratio of 1:1 for *P. falciparum* and *P. vivax* by both the thick blood smear and the QBC method, which is different from the 1:2 ratio reported for Colombia as a whole (Ministry of Health, unpublished data). As detected by thick smear, 0.4% of the patients were infected with *P. malariae,* which is also similar to the national incidence of this species.

The relative merits of the QBC method versus standard light microscopy have been controversial. Because of the cost and the requirement for a fluorescent microscope, it has not replaced the thick blood smear for the diagnosis of malaria in the field or in clinics.[26] In our study, the sensitivity of the thick blood smear was 91.11% when the QBC method was used as the gold standard. Other investigators have also reported an increased level of detection using the QBC method compared with the thick blood smear.[27,28] The specificity of these techniques did not differ. However, species identification is problematic with the QBC technique.[29] In our hands, *P. malariae* was scored as *P. vivax* in the QBC assay, and there was one case of a QBC method diagnosis of *P. falciparum* that had been identified as *P. vivax* by a thick blood smear. Furthermore, parasitemia cannot be easily quantified or can samples be stored for later examination.[29]

There was no difference in prevalence of *P. falciparum* or level of parasitemia diagnosed by thick smear in samples, which were analyzed by the PCR, versus those not assayed by this method, indicating that our subsampling for PCR analysis was unbiased.

TABLE 5

Comparison of malaria diagnosis of 3 methods in Buenaventura, Colombia*

	QBC +	QBC −	Total
Positive PCR			
Thick blood smear +	8	2	10
Thick blood smear −	1	69	70
Total	9	71	80
Negative PCR			
Thick blood smear +	10	0	10
Thick blood smear −	5	272	277
Total	15	272	287

* PCR = polymerase chain reaction; QBC = quantitative buffy coat.

Compared with the PCR, the sensitivity of conventional microscopy was lower than that reported by other investigators.[8,30] Our study population is in an area of low endemicity, where parasitemias and prevalence are typically low. Studies in other settings with higher endemicity may show greater sensitivity by microscopic techniques due to higher average levels of parasitemia. In previous studies, it was shown that sensitivity of microscopic examination tends to decrease compared with DNA-based detection methods when the incidence of positive cases decreases.[24,25] When the results of the QBC analysis are compared with a PCR gold standard, the sensitivity was only 9.78%. This is much lower than the relative sensitivity of microscopic detection versus the PCR in previously published studies,[8,25,30] and may be due to the low level of average parasitemia in our study population, and also might reflect sensitivity problems associated with delayed reading of our samples.

Similar to the comparison between the PCR and the QBC method, the PCR assay was much more sensitive than the thick blood smear. When the thick blood smear was compared with a PCR gold standard, the sensitivity of the microscopic technique was only 10.2%. Laserson and others[14] also found that PCR detection was much more sensitive, while Jelinek and others[31] found a multiplex PCR assay to be less sensitive than the thick blood smear. The oligonucleotide primers used here amplify repetitive sequences of high copy number, increasing the sensitivity of the PCR assay. Of the 12 PCR-negative, thick smear-positive samples of *P. falciparum,* 10 were also positive by the QBC method, indicating that the microscopists were probably correct; either the PCR results were false negatives or the microscopists had misidentified the species of parasite. As expected, the 17 *P. vivax* infections (diagnosed by microscopy) were negative by the PCR because the PCR primers are specific for *P. falciparum.*

Ten samples that were positive for *P. vivax* by thick blood smear also yielded a positive PCR signal, suggesting undetected mixed infections or a misdiagnosis by the microscopist, since the primers used in this study are specific for *P. falciparum* DNA.[12,14]

When all 3 diagnostic methods were compared, the PCR displayed the greatest level of detection. Of the 367 samples analyzed by all 3 techniques, 2.2% were positive by all 3 techniques, 74.1% were negative by all 3, 18.8% were positive by the PCR but negative by both microscopic techniques, and 2.5% were negative by the PCR but positive by both microscopic techniques. As stated above, 41 (59.4%) of the 69 PCR-positive samples showed weak signals, suggesting the ability of the PCR to detect low-level parasitemias that might not have been detected otherwise. To the best of our knowledge, this is the first report comparing all 3 diagnostic techniques on field-collected samples.

Our results confirm that PCR-based diagnosis is much more sensitive than microcopy-based techniques for malaria detection. We have implemented new techniques for public health surveillance and epidemiologic investigations on the Colombian Pacific coast. The PCR technique will improve our malaria diagnosis capability, as well as provide a means for tracking parasite strains and early identification of drug resistance. The enhanced capabilities should allow health professionals to develop better control measures and improve resource allocation based on a better understanding of prevalence of malaria in the area.

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