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Usefulness of Seminested Multiplex PCR in Surveillance of Imported Malaria in Spain

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The use of a new PCR-based method for the diagnosis of malaria in the Spanish Malaria Reference Laboratory has promoted an increase in confirmed cases of malaria. From August 1997 to July 1998, a total of 192 whole-blood samples and 71 serum samples from 168 patients were received from the hospitals of the Spanish National Health System. Most of the patients came from west-central African countries (85%). This molecular method showed more sensitivity and specificity than microscopy, detecting 12.4% more positive samples than microscopy and 13% of mixed infections undetectable by Giemsa stain. *Plasmodium falciparum* was the main species detected, with 68% of the total positive malaria cases, followed by *Plasmodium malariae* (29%), *Plasmodium vivax* (14%), and *Plasmodium ovale* (7%), including mixed infections in all cases. This report consists of the first wide, centralized survey of malaria surveillance in Spain. The reference laboratory conducted the analysis of all imported cases in order to detect trends in acquisition. The use of a seminested multiplex PCR permitted confirmation of the origins of the infections and the *Plasmodium* species involved and confirmation of the effectiveness of drug treatments. This PCR also allowed the detection of the presence in Spain of primaquine-tolerant *P. vivax* strains from west-central Africa, as well as the detection of a *P. falciparum* infection induced by transfusion.

Malaria has a major place among the endemic tropical diseases. Malaria risk of varying degrees existed in 100 countries and territories in 1994 (26). The increase of tourism and cooperation with developing countries and population migrations forced by wars and socioeconomic factors have caused an increase of malaria cases reported in immigrants and travellers returning from areas where the disease is endemic, with rates of case fatality up to 0.8% in countries like the United Kingdom (4) and Germany (1), mainly due to late or incorrect diagnosis. In Spain, malaria was eradicated in 1962, the last year of reported autochthonous cases. From 1985 to 1992, the number of imported malaria cases in Spain per year ranged between 100 to 150 and has progressively increased to 230 cases. Malaria is a notifiable disease in Spain, but supposedly only 30 to 40% of all cases are reported (15). This situation also exists in other developed countries, such as Switzerland or the United States, where only 25 to 50% of the cases are reported to health authorities (26).

Several molecular methods based on the amplification of DNA have been developed for the detection of malarial infections in humans (8, 9, 18, 20, 21, 25), but only one of these, using five PCRs, can differentiate between the four species of *Plasmodium* (20, 21). The malaria laboratory of the National Center for Microbiology in Spain is using a seminested multiplex malaria PCR (SnM-PCR) with just two PCRs, capable of detecting and differentiating between the four human malaria species in blood samples (16). Moreover, this method includes a positive amplification control in each sample to prevent false negatives. The incorporation of these molecular tools for the characterization of parasite infections has allowed an increase of sensitivity in the detection of human malarial parasites in blood, but these tests are presently only used in field trials.

The World Health Organization (WHO) recommends the surveillance of all imported malaria cases and the indirect surveillance of the spreading of malaria resistance in developing countries by the reference laboratories of developed countries. Therefore, these methodologies could increase the specificity and sensitivity of diagnosis and, if these methodologies are used in microbiology laboratories, could reduce the rate of case fatalities due to incorrect diagnoses.

The main objective of this work was to provide the Spanish National System of Health with a reference laboratory that surveilled imported malaria cases. The laboratory criterion for diagnosis is the demonstration of malarial parasites in blood films, and confirmation of cases is by PCR (as a complementary tool to traditional microscopy). Additionally, following the recommendations of the WHO, the reference laboratory analyzed the confirmed cases, determining the origins of the malaria infections and the *Plasmodium* species involved and following patients after treatment in order to evaluate the effectiveness of antimalarial drugs.

MATERIALS AND METHODS

The study, approved by the Ethical Committee of the Carlos III National Institute of Health, was carried out in the Parasitology Department of the National Center for Microbiology in Madrid, Spain. The main duty of the department is to provide fast and accurate diagnoses to the microbiology departments of state hospitals.

Patients, samples, and definitions of terms used. From August 1997 to July 1998, a total of 192 whole-blood samples and 71 serum samples from 168 patients

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with at least one symptom compatible with clinical malaria were received from 25 hospitals of the Spanish National Health System to be diagnosed for possible infection with *Plasmodium* spp. In several cases, different samples were sent from the same patients after confirmation of the malaria infection for surveillance of treatment effectiveness.

One hundred eight of 168 patients (64%) were semi-immune patients from countries where malaria is endemic. Fifty-nine patients (35%) were nonimmune Spanish travellers who had returned to Spain after visiting some country where malaria is endemic, and one patient was a Spanish woman who had never travelled overseas but who had received a blood transfusion.

The diagnosis of malaria by thin and thick blood smears, indirect fluorescent antibody test (IFAT), and PCR is given in less than 24 h so that physicians can apply the correct treatment as soon as possible. The laboratory criterion for diagnosis was the demonstration of malarial parasites in blood films, and diagnoses were confirmed by PCR. An episode of microscopically diagnosed and PCR-confirmed malaria parasites or malarial DNA from blood was defined as a confirmed case. Malaria acquired outside of Spain was described as imported malaria, and malaria acquired through artificial means (e.g., blood transfusion or use of shared syringes) was termed induced malaria. Renewed manifestations of malarial infection that were separated from previous manifestations of the same infection by an interval greater than the usual periodicity of the onset of paroxysms was defined as relapsing malaria. Finally, resistance was defined as the ability of a parasite to multiply or survive in the presence of a drug at a concentration that normally destroys parasites or prevents their multiplication. Classification of drug resistance was made according to the following WHO criteria: RI, parasitemia clears but the illness recurs (recrudescence) within 3 weeks; RII, reduction but not a clearance of parasitemia; and RIII, no reduction of parasitemia.

Thick and thin blood smears from whole-blood samples were stained with 10% Giemsa stain (Merck, Darmstadt, Germany) and were examined microscopically by two qualified technicians. Level of parasitemia per microliter of blood was measured as parasite count = (leukocyte count × parasites measured per 100 leukocytes)/100, where the leukocyte count was 4,000.

IFAT for the presence of malarial antibodies were performed with schizonts from cultures of the 3D7 clone of *Plasmodium falciparum*. IFAT was used mainly in nonimmune people (travellers) and for estimation of total antibodies in *P. falciparum* infections. This test uses *P. falciparum* homologous antigen, and for this reason the test is of particular value for the detection of *P. falciparum* antibodies in as little as several days after the appearance of malaria parasites in blood and for the screening of blood collected for blood banks.

PCR test. The method of DNA extraction (from 3 μl of blood) was carried out by the Chelex method with minor modifications (16, 24). Detection and identification of malarial species were simultaneously performed with a sequence of two SnM-PCRs (16), and the sizes of the products were estimated after electrophoresis on a 2% agarose gel and staining with ethidium bromide.

The PCR mix for the first reaction consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 1% glycerol, a 200 μM concentration of each of the deoxynucleoside triphosphates, the PCR primers, 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer), and 41.1 μl of Chelex DNA as template in a final volume of 50 μl. The amounts of primers were 25 pmol for UNR (5′-GACGGTATCTGATCGTCTT-3′), 25 pmol for PLF (5′-AGTGTGTATCAATCGAGTTT-3′), and 1.25 pmol for HUF (5′-GAGCCGCCTGGATACCG-3′). This first reaction was expected to yield two products: the first being a band of 231 bp from UNR-HUF produced by the amplification of the small subunit of the human ribosomal gene (ssrDNA), the positive control for each individual sample, and the second being a band of 783 to 821 bp (depending on the *Plasmodium* species) from UNR-PLF that should detect the presence of any malaria species by amplification of the ssrDNA of *Plasmodium* spp. The UNR-HUF fragment is the individual positive control, and it must be present in every sample. The absence of this amplification product shows that the PCR was inhibited and the result of the second PCR must be ignored in order to avoid false negatives.

The reaction mix for the second PCR (SnM-PCR) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 1% glycerol, a 200 μM concentration of each of the deoxynucleoside triphosphates, the PCR primers, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer), and 4 μl of a 4/1,000 dilution of the PCR product of the first reaction as template in a final volume of 25 μl. The concentration of primers was 25 pmol for PLF, 3.12 pmol for MAR (5′-GCCCTCCAATTGCCTTCT-3′), 15 pmol for FAR (5′-AGTTCCCTAGAATAGTTACA-3′), 6.25 pmol for OVR (5′-GCATAAGGAATGCAAAGAACAG-3′), and 2.5 pmol for VIR (5′-AGGACTTCCAAGCCGAAG-3′). Infections with different human *Plasmodium* species yield products of different sizes. A band of 269 bp indicates *Plasmodium malariae* infection, a band of 395 bp shows *P. falciparum* infection, a band of 436 bp suggests a *Plasmodium ovale* infection, and a band of 499 bp indicates a *Plasmodium vivax* infection. Mixed infections would show the appropriate bands.

For both reactions, a 2400 GeneAmp PCR system (Perkin-Elmer) thermal cycler was used, beginning with a 5-min denaturation at 94°C, followed by (first reaction) 40 cycles of 45 s at 94°C, 45 s at 62°C, and 60 s at 72°C or (SnM-PCR) 35 cycles of 20 s at 94°C, 20 s at 62°C, and 30 s at 72°C. The final cycle was followed by an extension time of 10 min at 72°C.

In order to avoid possible contamination, separate space was designated for the setup of different PCRs and the storage of filter tips, etc., and several negative controls (no DNA, uninfected blood, infected *Rattus* blood with *Plasmodium bergei*, and no extracted DNA) and positive controls for each *Plasmodium* infecting humans were used in order to localize any possible contamination.

RESULTS

Western or central African countries were the most common points of departure for malaria patients examined in this study (84% of patients) followed by Asia (8.5% of patients). Eastern African and Central American countries were the origin of 7% of the cases.

Sixty-one of 108 patients (56.5%) born in countries where malaria is endemic tested positive by SnM-PCR, while microscopy detected 51 infected individuals (47%). Twenty-seven of 59 (45.7%) nonimmune Spanish travellers tested positive by SnM-PCR, while microscopy detected 19 (32.2%) patients with malarial asexual parasites in their blood. The induced malaria (the Spanish woman who received the blood transfusion) corresponded to a *P. falciparum* infection by the SnM-PCR, and 10 days later she also tested positive by IFAT but continued to test negative by microscopy. In total, 89 of 168 patients (53%) tested positive by SnM-PCR, while 70 patients (41.7%) tested positive for malaria by microscopy. SnM-PCR proved capable of detecting malarial parasites in 11.3% of infected patients who had tested negative by microscopy of thin and thick films.

A total of 24 additional blood samples from 14 malaria patients were assayed to confirm the effectiveness of the treatment. SnM-PCR still showed the presence of malarial parasites in 11 (45.8%) of these cases, while microscopy detected malaria infection in six cases (25%). This result is 20.8% less effective than the detection of parasites by the amplification of DNA by PCR.

In global terms, the SnM-PCR detected 100 of 192 (52%) malaria infections, while microscopy detected 76 (39.6%) positive samples, indicating that SnM-PCR detected 12.4% more malaria infections than did microscopy (Table 1).

The most prevalent *Plasmodium* species in the samples, considering the SnM-PCR results, was *P. falciparum* (68%), which was present in 59 samples as a single infection and in nine other cases as a mixed infection. *P. malariae* was detected by itself in 16 samples (20%) and in five mixed samples. This data confirms that quartan malaria was the second most prevalent infection detected, followed by *P. vivax* (14%) and *P. ovale* (7%), which were present in 10 and 6 samples, respectively, as single infections and were present in four and one samples, respectively, as mixed infections. The SnM-PCR was able to identify nine mixed infections, while microscopy detected two (detection of 13% more mixed infections by PCR than by microscopy). All the mixed infections detected were from people born in countries where the disease is endemic, except one triple infection which was from a Spanish missionary who had lived in western Africa, Asia, and South America over the last 20 years.

In 135 of 192 blood samples, the SnM-PCR and microscopy showed the same results, including in the case of the triple infection. However, in 36 samples, SnM-PCR detected malarial species not identified by microscopy. Out of 116 microscopically negative samples, 30 were SnM-PCR positive, and SnM-PCR identified a second *Plasmodium* species in six other samples (Table 2). The only discrepancy resolved in favor of microscopy was a mixed infection of *P. falciparum* and *P. ovale* in which only the *P. falciparum* was detected by SnM-PCR. In this case, the positive control of the first reaction was partially inhibited, probably due to the fact that the sample was of clotted blood.

IFATs presented discrepancies with the data obtained by

TABLE 1. Patients, samples, and results divided by social groups and results of SnM-PCR and microscopy

Patient group	Initial test				Secondary test[a]				Total samples
	No. of samples	Method	No. of samples positive (%)	No. of samples negative (%)	No. of samples	Method	No. of samples positive (%)	No. of samples negative (%)	
Immigrant	108	PCR	61 (56.5)	47 (43.5)	9	PCR	6 (66.6)	3 (33.3)	117
		Microscopy	51 (47)	57 (53)		Microscopy	4 (44.4)	5 (56.6)	
Traveller	59	PCR	27 (45.7)	32 (54.3)	14	PCR	5 (35.7)	9 (64.2)	73
		Microscopy	19 (32.2)	40 (67.8)		Microscopy	2 (14.3)	12 (85.7)	
Spanish native	1	PCR	1 (100)	0 (0)	1	PCR	0 (0)	1 (100)	2
		Microscopy	0 (0)	1 (100)		Microscopy	0 (0)	1 (100)	
Total	168	PCR	89 (53)	79 (47)	24	PCR	11 (45.8)	13 (54.2)	192
		Microscopy	70 (41.7)	98 (58.3)		Microscopy	6 (25)	18 (75)	

[a] Samples resubmitted for evaluation of the effectiveness of treatment with antimalarial drugs.

microscopy and SnM-PCR. In semi-immune people, six samples (8%) with serological titers higher than 1/10,240 and 8 (11%) with titers higher than 1/5,120 tested negative by both SnM-PCR and microscopy. In these individuals, antimalaria antibodies may still be detectable many months or years after detectable parasitemia. On the other hand, nonimmune people present antibodies in as little as several days after the appearance of malarial parasites in the blood. In accordance with this fact, only two (3%) of the total of IFAT-negative samples tested positive by both SnM-PCR and microscopy, corresponding to nonimmune individuals with a primary malarial infection.

Two siblings born in Equatorial Guinea, a boy and a girl (4 and 5 years old, respectively), tested positive by microscopy and SnM-PCR a few days after they returned to Spain. In the hospital, both children tested positive for infection by *P. falciparum* by microscopy and began the standard treatment of 7 days of quinine sulfate and clindamycin. In the reference laboratory, PCR determined that both children had a mixed infection with *P. falciparum* and *P. vivax*. Once the first treatment was finished, SnM-PCR detected a single *P. falciparum* infection in the boy, while the girl tested negative by SnM-PCR. This result demonstrates the failure of seven days of treatment with quinine sulfate and resulted in the start of a new course of treatment with quinine for 10 days and without treatment with primaquine diphosphate. Forty days later, the children tested positive for *P. vivax* infections by SnM-PCR. Afterwards, the children were treated with primaquine diphosphate (0.25 mg/kg of body weight) for 14 days. One month later, new samples showed that the boy was infected by *P. vivax* only. Higher dosages of primaquine diphosphate (0.35 mg/kg of body weight for 14 days) together with a new treatment of quinine sulfate for 7 days was used for treatment of this relapse. Three months later, after the appearance of new clinical malaria in the boy, a blood sample tested positive for *P. falciparum* infection by microscopy and a *P. falciparum* and *P. vivax* infection by SnM-PCR. The boy was then treated with mefloquine (15 mg per kg of body weight) plus a higher dosage of primaquine diphosphate (0.5 mg/kg of body weight for 14 days). After this treatment, clinical cure and absence of parasites in the blood were confirmed by microscopy and SnM-PCR.

The sample from the 63-year-old Spanish woman who presented symptoms of clinical malaria but never travelled outside Spain was confirmed as being a *P. falciparum* infection by the SnM-PCR. This case of induced malaria resulted from a blood transfusion from a patient with a history of malarial infection 7 months earlier. Retrospective analysis of the sera confirmed the acquisition of malarial infection in the following way: (i) a sample of serum dated prior to the transfusion tested negative for infection by IFAT (titer $< 1/80$), (ii) serum collected 20 days posttransfusion resulted in a positive titer of 1/2,560, and (iii) 1 month later the IFAT titer was 1/5,160.

DISCUSSION

This study of the sources of malarial infections detected in Spain indicates that west-central Africa is a major risk area for acquiring malarial infections. Among the samples with origins of infections in west-central Africa, the majority were from Equatorial Guinea, mainly due to the high rate of emigration from that country as well as the Spanish governmental collaboration with this former Spanish colony. The high number of malarial infections in Spain that originate from this country is explained by the high malaria morbidity (3).

P. falciparum is present in the 68% of the cases of imported malaria. *P. malariae* (20%) was the second most common species in these cases, followed by *P. vivax* (mainly from Asian and Central American regions) and *P. ovale* (which is restricted to west-central Africa).

SnM-PCR increased the detection of malarial parasites, including in cases of mixed infections. Data about mixed infections in which *P. vivax* and *P. ovale* are the second species involved makes clear the importance of correct diagnosis of the specific infection and the correct treatment of the liver parasite stages in order to prevent relapses.

IFAT is a good diagnostic method for people who have not

TABLE 2. Matrix comparing results of SnM-PCR with those of microscopy[a]

Microscopy result	No. of samples with PCR result									Total
	Neg	F	M	O	V	FM	FO	FV	FMV	
Neg	86	20	8	1	1					116
F	1	29		1		2		3		36
M		1	7	1		1				10
O				3						3
V					9					9
FO		1								1
FMV									1	1
Psp	5	8	1			1	1			16
Total	92	59	16	6	10	4	1	3	1	192

[a] Neg, negative; F, positive for *P. falciparum*; M, positive for *P. malariae*; O, positive for *P. ovale*; V, positive for *P. vivax*; Psp, *Plasmodium* species not determined.

been in previous contact with the infection. A few days after the blood is invaded, antibodies to erythrocytic asexual stages become detectable by the more sensitive tests. A few days later, high levels of antimalarial antibodies are reached, and these levels persist after the parasitological crisis has concluded. In this study, IFAT provided useful complementary information on several nonimmune patients and should be considered as a combined indicator of point prevalence and recent period prevalence of the disease. This data was confirmed for *P. falciparum* infections, because homologous *P. falciparum* antigen was used during IFATs. Although homologous *P. falciparum* antigens were used on IFATs, some cross-reactions were presented on samples when other species were involved in malaria infections.

The case of malaria induced by blood transfusion was detected by SnM-PCR and confirmed by IFAT and shows the necessity of using PCR or other molecular diagnostic methods to screen blood donors for possible malaria infection. IFAT could be adequate for persons who have had malaria in the past, although the test does not always indicate the actual presence of infection. In developed countries, serological testing is the current methodology to detect antimalarial antibodies during the screening of blood (5), but in countries where the disease was formerly endemic, where number of malaria cases increases every year due to immigration, tourism, etc., other methods of direct detection could be used. Regulations governing the acceptance of whole blood for transfusion vary considerably from one country to another, and it would be desirable for some internationally acceptable criteria to be established. PCR has been previously used for the screening of blood donors and demonstrates more sensitivity than microscopy (20, 23) and should be a useful tool to investigate potentially dangerous blood donors with histories of malaria or exposure to the infection.

Two mixed infections of *P. falciparum* plus *P. vivax* in two natives of Equatorial Guinea, a west-central African country where the majority of the population lacks the Duffy receptor necessary for the invasion of the erythrocyte by *P. vivax* (10, 11), confirmed the presence of a focus of *P. vivax* (16) in that country. Occurrences of *P. vivax* relapses in these patients after primaquine therapy would be assumed to be the most reliable indication of resistance. In this case, the failure of initial and secondary treatments was demonstrated by the detection of *P. vivax* by SnM-PCR, an infection accompanied by clinical features. The administration of higher dosages of primaquine (7, 19) after the third relapse gave good results and prevented any further malaria symptoms. The time of the appearance of the relapses (8 to 12 weeks) could be indicative of a short-term strain type, like some strains from southeast Asia (6). Until now, primaquine-tolerant strains have been described in southeast Asia (14, 22), the western Pacific, and Central America (12, 17), but only sporadic cases have been described in eastern Africa (13). It must be noted that the infection of this patient by *P. falciparum* was also resistant to standard treatment. In west-central Africa, an infection of two resistant malarial parasites (quinine-resistant *P. falciparum* and primaquine-resistant *P. vivax*) in the same individual had not been previously reported (22). This could suggest that the mixed infection originates in some Asiatic areas and was imported to west-central African countries where Asiatic populations have been increasing during recent decades.

Furthermore, three other cases of *P. falciparum* strains tolerant to quinine were found, two from Equatorial Guinea and one from Pakistan. The percentage of quinine-tolerant strains is still very low (7.3%) in comparison with chloroquine and mefloquine resistances, but the number of resistant strains is increasing (26), and in the future it could be necessary to develop new strategies for malaria therapies.

The appearance of a possible autochthonous case of *P. vivax* in Italy (2) and the increase of malaria cases in north Africa (27) have necessitated the establishment of a system of malaria surveillance in Spain. The risk of the reintroduction of malaria to countries where the disease has been eradicated is minimal (26) due to socioeconomic development and the high level of health care, but a system of surveillance of reemerging diseases, like malaria, needs to be established.

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