

Title: Surveillance of imported malaria in Spain: The useful tool of the Semi-Nested Multiplex PCR

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Abstract

The use of a new PCR based method for the diagnosis of malaria in the Malaria Reference Laboratory has promoted an increase in suspected malaria cases from the Spanish Hospitals. From August 1997 to July 1998, a total of 192 whole blood samples and 71 sera 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 in comparison to microscopy detecting 12.4% more positive samples than microscopy and 13% of mixed infections undetectable by Giemsa stain.

P.falciparum was the main specie detected with 68% of the total positive malaria cases following of *P.malariae* (29%), *P.vivax* (14%) and *P.ovale* (7%), including mixed infections in all cases.

Until now no tolerant *vivax*-strain had been reported from West-Central Africa. PCR detected two *vivax*-infections primaquine tolerant strains from this region and a *falciparum*-infection by blood transfusion in a Spanish woman.

Introduction

Malaria has a major place among the endemic tropical diseases. Malaria risk of varying degrees existed in 100 countries and territories [1]. The increase of tourism, the non governmental collaboration with developing countries and mainly migration by wars and socio-economic factors have caused an increase of malaria imported cases in immigrants and travellers returning from endemic areas to malaria-free countries. In Spain malaria was eradicated in 1962, the last year of reported cases. Since 1960s all malaria infections reported have been imported cases. From 1985 to 1992 the number of malaria imported cases per year ranged between 100 and 150 and, actually have progressively increased up to an average of 230 cases per year, but just the 30-40% are reported [2]. WHO recommends the surveillance of all imported malaria cases and positive action by European laboratories in the indirect surveillance of the spreading of malaria resistance in developing countries.

The incorporation of the molecular tools for the diagnosis of parasite infections has allowed an increase of sensitivity in the detection of human malaria parasites in blood. The malaria laboratory of the National Centre of Microbiology in Spain is using a semi-nested multiplex malaria PCR (SnM-PCR) capable of detecting the four human malaria species in blood samples. Nowadays, various molecular methods based on the amplification of DNA have been developed for the detection of the malaria infections in humans [3-8], but only one of these can detect the four species of *Plasmodium* [6-7], using five PCR reactions in contrast to the two steps of the SnM-PCR [9]. Moreover, the present method includes an individual positive control in each sample to prevent false negatives.

The aim of this work was: a) carry out the study of the malaria surveillance in Spain, including origin of infections, *Plasmodium* species involved and possible susceptibility to traditional treatments and b) to compare the SnM-PCR with the traditional Giemsa stain diagnosis

method, in order to improve the accuracy and the sensitivity of the diagnosis using this new methodology and, the following of the patients after treatment of clinic episodes in order to evaluate the effectiveness of antimalarial drugs.

Material and Methods

The study was carried out in the Parasitology Department of the National Centre of Microbiology in Madrid from August 1997 to July 1998. A total of 192 whole blood samples and 71 sera were received from the National Health System (INSALUD) from 168 patients with at least a symptom compatible with clinic malaria. In several cases different samples were sent from the same patients for surveillance of effectiveness of the treatment.

108 patients (64%) correspond to semi-immune patients from malaria endemic countries. 59 patients (35%) were non-immune Spanish travellers, who come back after having visited some endemic malaria countries, and one patient was a old Spanish woman, which never travel overseas.

The last endemic malaria country visited for the patients before coming back to Spain was in 141 cases (84%) a West or Central African country (126 cases from Equatorial Guinea, former Spanish colony), 14 cases (8%) from Asia (mainly from India) and the resting 12 cases (7%) were from Eastern Africa and Central America.

Whole blood samples were collected, thick and thin blood smears stained with 10% Giemsa (Merck, Germany) and examined microscopically by two technicians. Immuno Fluorescence Assay (IFA) tests were performed in our laboratory from cultures of the 3D7 clone from a strain of *P.falciparum*.

The extraction method of DNA (from 3µl of blood) was carried out using the Chelex method with minor modifications [9,10].

Detection and identification of malaria species were simultaneously performed using a

sequence of two (semi-nested multiplex) PCR reactions [9], and the size of products were estimated from 2% agarose gel electrophoresis using ethidium bromide staining .

The PCR reaction mix for the first reaction consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (W/V) gelatin, 1% glycerol, 200 µM each of dNTPs, the PCR primers, 2.5 units AmpliTaq DNA polymerase (Perkin-Elmer, NJ, USA) and 41.1 µl of template DNA in a final volume of 50 µl. The concentration of primers were 25 pmoles for UNR (5'-GACGGTA TCTGATCGTCTT-3'), 25 pmoles for PLF (5'-AGTGTGTATCAATCGAGTTT-3') and 1.25 pmoles for HUF (5'-GAGCCGCCTGGATACCG-3'). This first reaction is expected to yield two products: the first is a band of 231 bp from UNR-HUF produced by the amplification of human *ssrDNA* (the positive control for each individual sample) and a second band of 783-821 bp (depending on the *Plasmodium* species) from UNR-PLF that should detect the presence of any malaria species, although this fragment is not visible on the agarose gel. In the multiplex-PCR the reaction mix consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.001% (W/V) gelatin, 1% glycerol, 200 µM each of dNTPs, the PCR primers, one unit AmpliTaq DNA polymerase (Perkin-Elmer) and 4 µl of a dilution 4/1000 of the PCR product of the first reaction as template in a final volume of 25µl. The concentration of primers, empirically obtained, was 25 pmoles for PLF, 3.12 pmoles for MAR (5'-GCCCTCCAATTGCCTTCT-3'), 15 pmoles for FAR (5'-AGTTCC CCTAGAATAGTTACA-3'), 6.25 pmoles for OVR (5'-GCATAAGGAATGCAAAGAACAG-3') and 2.5 pmoles for VIR (5'-AGGACTTCCAAGC CGAAG-3'). Infections with different human *Plasmodium* species yields products of different sizes. A band of 269 bp indicates *P.malariae* infection; a band of 395bp shows *P.falciparum* infection; a band of 436 bp suggests a *P.ovale* infection and a band of 499 bp indicates a *P.vivax* infection. Mixed infections would show all the appropriate bands.

For both reactions a 2400 GeneAmp PCR system (Perkin-Elmer, USA) thermal cycler was used,

beginning with five minutes denaturation at 94°C, followed by (first reaction) 40 cycles with 45 seconds at 94°C, 45 seconds at 62°C, and 60 seconds at 72°C or (multiplex-PCR reaction) 35 cycles with 20 seconds at 94°C, 20 seconds at 62°C and 30 seconds at 72°C. The final cycle was followed by an extension time of ten minutes at 72°C.

Results

89 patients (53%) were malaria positive by the SnM-PCR while, just, 70 patients (41.7%) were malaria positive by microscopy. SnM-PCR proved capable of detecting 11.3% of infected patients that were negatives on thin and thick films by microscopy. A total of 24 new blood samples from several diagnosed patients were sent, after total or partial treatment, to confirm the effectiveness of the treatment. SnM-PCR, still, show the presence of malaria parasites in 11 cases (45.8%) while the microscopy just detected a malaria infection in 6 cases (25%), 20.8% less than the amplification of DNA by PCR. The SnM-PCR detected 100 (52%) malaria infections of the total of processed samples (192), while microscopy detected 76 (39.6%) positive samples, indicating 12.4% more of detected malaria infections than by microscopy (Table 1).

By social groups, 61 patients (56.5%) born in endemic countries where malaria is an endemic disease were positive by SnM-PCR while microscopy just detected 51 infected individuals (47%). Twenty seven (45.7%) non-immune Spanish travellers were positive by the SnM-PCR, while the microscopy detected 19 (32.2%) patients with malaria asexual parasites in blood. The old Spanish woman was positive by the SnM-PCR and ten days later was positive also by IFA test but was negative by microscopy (Table 1).

The most represented *Plasmodium* species in the samples, considering the SnM-PCR results, was *P.falciparum* (68%), which is present in 59 samples as a single infection and in other 9 as a mixed infection. *P.malariae* with 16 positive samples (20%) plus 5 mixed infection samples

resulted the second *Plasmodium* specie more often detected followed of *P.vivax* (14%) and *P.ovale* (7%), which are present in 10 and 6 samples respectively as single infections and 4 and 1 respectively as mixed infections. The SnM-PCR was able to identify 9 mixed infections while the microscopy just detected two (13% more of mixed infections by PCR than the microscopy). All the mixed infections detected were from people who have born in endemic countries except a triple infection from a Spanish missionary who lived in West-Africa, Asia and South-America in the last twenty years (table 2).

In 135 out of 192 blood samples the SnM-PCR and the microscopy show the same results, including a triple infection. However, in 36 samples the SnM-PCR detected malaria species not identified by microscopy. Out of 116 microscopically negative samples 30 were SnM-PCR positive and in other 6 cases SnM-PCR identified a second *Plasmodium* specie in the sample. When different results of PCR and microscopy where obtained, the thin and thick films were carefully re-examined resolving 84% of all discrepancies in favour of the SnM-PCR (table 3).

The only discrepancy resolved in favour of the microscopy was a microscopically mixed infection *P.falciparum* plus *P.ovale* from which only *P.falciparum* was detected by the SnM-PCR. In this case, the positive control on the first reaction was partially inhibited, probably, due to the fact that the sample was clotted blood. In 9 cases the discrepancy remained unresolved but probably due to a higher sensitivity of the SnM-PCR method than to a missed diagnosis (table 3).

According to expected results IFA serological tests presented discrepancies with the data obtained by microscopy or by SnM-PCR. Just 30 (42%) or 43 (60.5%) samples in comparison to microscopy or to SnM-PCR respectively show equal positive or negative results. Six samples (8%) with titre higher than 1/10240 and 8 (11%) with titres higher than 1/5120 were negatives by SnM-PCR and microscopy while 2 (3%) samples IFA negatives were positive by SnM-PCR and microscopy.

New samples (24) from several patients (14) previously diagnosed by SnM-PCR were sent to confirm the total elimination of the parasite in blood or because the patient still had malaria symptoms after partial or total treatment.

Two brothers born in Equatorial Guinea, a boy and a girl (4 and 5 years of age respectively), were analysed by microscopy and SnM-PCR few days after they came back to Spain. Both children presented a *falciparum*-infection by microscopy beginning standard treatment with seven days of quinine sulphate and clindamicine. PCR determined in both (girl and boy) a mixed infection by *P. falciparum* and *P. vivax* in our laboratory. On this date both were treated with quinine sulphate at guideline of 10 days. Once finished the first treatment with quinine sulphate (10 days) and clindamicine is received a new sample of both children determining by SnM-PCR a single *falciparum*-infection in the boy being parasite negative the girl. This result demonstrates the failure of the treatment with quinine sulphate at guideline of ten days, beginning a new treatment with quinine during 10 days and no receiving treatment with primaquine diphosphate. Forty days later two new samples of the two children were received in our laboratory resulting both *vivax*-malaria infections by PCR and the blood of the girl was also diagnosed like *vivax*-infection by microscopy. This result indicates a first relapse caused by the hepatic hypnozoites. The childrens were treated with primaquine diphosphate (0.25 mg/kg of weight during 14 days) after determination of negativity to G6PD-deficiency by filter paper blood spot fluorescence test. Three months later a new sample resulted a *vivax*-infection in the boy being malaria negative infection in the girl, resulting a new relapse caused by a tolerant strain to primaquine diphosphate. New guideline with primaquine diphosphate was used for treatment of this *vivax*-infection (0.35 mg/kg body weight /14 days) joint to a new treatment with quinine sulphate of seven days. Three months later is received a new blood sample of the boy resulting a *falciparum*-infection by microscopy and a *falciparum*+*vivax*-infection by SnM-PCR. Then, was carried out a medical prescription with mefloquine (15 mg per body

weight on twice) plus a new treatment with a higher dose of primaquine diphosphate (0.5 mg/kg of body weight/14 days) . After treatment, clinical cure and no presence of parasites in blood was confirmed by microscopy and SnM-PCR. At present, surveillance of this patient is being carried out for possible relapses.

A sample from a 63 years old Spanish woman who presented symptoms of clinical malaria but never travelled outside the country was confirmed as *P.falciparum* infection by the SnM-PCR. The woman was infected by blood transfusion from a patient with previous history of malaria infection seven months early. The serum of the patient previous to the transfusion resulted negative by the IFA test (<1/80). New serum twenty days after transfusion resulted positive at titre 1/2560 and one month later the serum was IFA positive at titre 1/5160.

Discussion

In 1997 the Malaria Reference Laboratory was created under the direction of the Parasitology Department of the Instituto de Salud Carlos III in Spain, with the main objective of carrying out the surveillance of the malaria imported cases suspected in the National Health System of Spain. The creation of this initiative has been well accepted by the Hospitals and Centres of the National System of Health in the country and has promoted a great perception by the departments of infectious diseases as well as the medical personnel in primary health care. One year later, more than 50% of malaria cases detected in Spain were diagnosed in this laboratory.

The main source of infection detected in Spain indicates to West-Central Africa as the major risk area for acquiring the malaria infections. Among the West-Central African samples studied, the majority of them were from Equatorial Guinea, mainly due to the high rate of immigration into Spain, as well as governmental and no governmental collaboration in the development of the

country joint to the business relations between Spain and this former Spanish colony. The high number of malaria infections from this country is explained by the high rates of malaria prevalence (80% of children under 10 years of age present parasites in blood) [11]. West-Central Africa corresponds to meso-hyperendemic areas where the socio-economic status of the population causes great difficulties in the implementation of the control activities by the National Malaria Programmes [1].

Seventy eight percent of the malaria positive patients show *Plasmodium falciparum* as source of the disease, and it could be imported from any endemic malaria region. *Plasmodium malariae* (20%) was the second specie involved following of *Plasmodium vivax* from Asian and Central American regions. *Plasmodium ovale* was restricted to West-Central Africa region. IFA serological test is just a good diagnosis method for people who have never been previously in contact with the infection, because semi-immune individuals present a acquired immunity after a first or repeat contact with the parasite producing IgG malaria antibodies that are present in the serum when no infection is present.

The SnM-PCR used in the laboratory shows to be a good tool in the detection and correct malaria diagnosis, in all cases, after correct treatment the malaria symptoms of the patients disappear, proving the accuracy of the diagnosis. Besides, PCR increased the detection of malaria parasites as regards to microscopy, allowing an increase in the patency level of parasite detection. 12.4% of negative cases by microscopy were positive samples when diagnosed by SnM-PCR and other 13% were diagnosed as double infections whereas the microscopy just detected single infections. This fact could be due to low parasitaemias in semi-immune patients due to acquired immunity and in no-immune patients determined for the prophylactic protection with malarial drugs.

Only two mixed infections could be detected by microscopy whereas SnM-PCR detected 8 mixed infections undetected microscopically, but missed one, probably for inhibition of the PCR reaction. Four of these mixed infections presented *P. falciparum* plus *P. vivax* or *P. ovale* species, indicating the importance of the determination of the *Plasmodium* specie or species involved in the infection for the specific treatment of the hepatic hypnozoites. This fact joint to the severity and development of the disease depending on the *Plasmodium* species involved.

Two mixed infections of *P. falciparum* plus *P. vivax* confirmed the presence of a possible focus of *Plasmodium vivax* in Equatorial Guinea [9], a West-Central African country with a majority population without the Duffy receptor, necessary for the *P. vivax* invasion of the red blood cell and opens news perspectives about of the consolidation of the transmission of this parasite in West and Central African populations. *Plasmodium vivax* infections were detected in samples from mulatto children. As the Fy allele does not produce neither Fy^a nor Fy^b antigens, Fy^{a-b-} phenotype is the major allele in Blacks (frequency: 0.7 to 1), and it is possible that a focus exists composed of white and mulatto individuals which maintain the transmission. Future studies on a possible correlation between the expressed Fy allele and the infection by *P. vivax* may explain the infections detected in Equatorial Guinea [12-13].

The occurrence of relapses of *P. vivax* after primaquine therapy would be assumed to be the most reliable indication of resistance. In this case failure to 1st (0.25 mg/kg/14 days) and 2nd (0.35 mg/kg/14 days) treatment, at different and increasing therapeutic doses, was demonstrated by the presence of *P. vivax* relapse detected by SnM-PCR accompanied by clinic features. The time of the appearance of the relapses (each 2,5-3 months) could be indicative of a strain type Cheson from Southeast Asia [14]. A few reports shown the presence of relapses in Africa indicating the Cheson pattern, but always on East-Africa not in West-Central Africa. Higher dose of primaquine was administered on the 3rd relapse (0.5mg/Kg/14 days) preventing the appearance until now of new

relapses. Higher doses of primaquine have been used by several authors for the treatment of hypnozoites in liver [15-16]. Until now, primaquine tolerant strains have been described in Southeast Asia [17-18], Western Pacific and Central America [19-20] but only sporadic cases of failure have been described in Eastern Africa [21]. It must be noted that *falciparum*-infection also was resistant to standard treatment, as seen after the third relapse of the disease where *P. falciparum* was also detected, even if the patient had not left Spanish territory in the meantime. This fact could be explained for low parasitaemias presented by semi-immune individuals which can persist during several months until appearance of a new clinical episode. The presence of *P. vivax* infection is very rare and the *P. ovale* infection rates are quite low in Equatorial Guinea, thus, the primaquine is not the current treatment choice in a case of clinic malaria. Besides, this fact is increased by the high rate of G6PD-deficient people because enzyme deficient people and pregnant women cannot use this antimalarial drug. This could suggest that this mixed infection (*P. falciparum* plus *P. vivax*) could have its origin in some Asiatic strains, imported into Equatorial Guinea. In Asia, prevalence of tolerant strains for both *Plasmodium* species is quite high, whereas in West-Central Africa a double resistance in the same individual had not been detected before [18].

Furthermore, there are other three cases of *P.falciparum* tolerant to quinine, two from Equatorial Guinea and the third from Pakistan. The percentage of tolerant strains to quinine is still very low 7.3%, in comparison to resistance at chloroquine and mefloquine, but the number of resistant strains is increasing [1].

A case of malaria by transfusion was detected by SnM-PCR and confirmed by IFA test in a 63 year old woman without any history of travel to endemic areas. Changes in population demographics, immigration, as well as increase in travel to endemic areas could contribute to increase the number of prospective blood donors who could be infected by malaria. PCR has been used in the screening of blood donors demonstrating more sensitivity than microscopy [22]. In

developed countries, serology is the most common technique to detect anti-malarial antibodies in the screening of blood donors. In the future, SnM-PCR will allow an increase in the sensitivity of the conventional diagnosis, avoiding infections by blood transfusion. PCR together to serology would be the most effective combination to detect malaria parasites in blood donors individuals [23].

Moreover, the appearance of a possible autochthonous case of *P. vivax* in Italy [24], the increase of malaria cases in North Africa [25] and the high rate of migration through Spain from Subsaharian individuals indicates the relevance to develop a system of surveillance capable of studying not only the clinical cases, but also the actual role of the *Anopheles* vectors and its capacity in malaria transmission in former Spanish endemic areas, in order to control the possible re-introduction of the malaria disease.

Some recent epidemics have been linked to climatic change in conjunction with socio-economic degradation, wars and collapse of health services. The risk for reintroduction of malaria in eradicated countries is minimal [1] due to socio-economic development and the high level of the Health System but a system of surveillance of re-emergence diseases like malaria due be established in Spain.

Acknowledgements

This work has been supported by the Fondo de Investigaciones Sanitarias (FIS) contract number 96/0216 and the Spanish Agency of International Cooperation.

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Table 1. Patients, samples and results by social groups and totals by SnM-PCR and microscopy.

	Patients	Method	Positives	Negatives	Repeated* samples	Method	Positives	Negatives	Total samples	Method	Positives	Negatives
Immigrants	108	PCR	61 (56.5%)	47 (43.5%)	9	PCR	6 (66.6%)	3 (33.3%)	117	PCR	67 (57.3%)	50 (42.7%)
		Micro.	51 (47%)	57 (53%)		Micro.	4 (44.4%)	5 (56.6%)		Micro.	55 (47%)	62 (53%)
Travellers	59	PCR	27 (45.7%)	32 (54.3%)	14	PCR	5 (35.7%)	9 (64.2%)	73	PCR	32 (43.8%)	41 (56.2%)
		Micro.	19 (32.2%)	40 (67.8%)		Micro.	2 (14.3%)	12 (85.7%)		Micro.	21 (28.8%)	52 (71.2%)
Spanish native	1	PCR	1 (100%)	0 (0%)	1	PCR	0 (0%)	1 (100%)	2	PCR	1 (50%)	1 (50%)
		Micro.	0 (0%)	1 (100%)		Micro.	0 (0%)	1 (100%)		Micro.	0 (0%)	2 (100%)
Totals	168	PCR	89 (53%)	79 (47%)	24	PCR	11 (45.8%)	13 (54.2%)	192	PCR	100 (52%)	92 (48%)
		Micro.	70 (41.7%)	98 (58.3%)		Micro	6 (25%)	18 (75%)		Micro.	76 (39.6%)	116 (60.4%)

*Samples received for the control of the effectiveness of the treatment with antimalarial drugs

Table 3. Discrepancies between SnM-PCR and microscopy before and after re-examination of the slides. = equal results. <> discrepancy on *Plasmodium* specie. -/+ Negative result by microscopy and single infection by PCR. +/+ Single infection by microscopy and double infection by PCR. ?/+ Undetermined *Plasmodium* infection by microscopy and determined single infection by PCR. ?/+ Undetermined *Plasmodium* infection by microscopy and determined double infection by PCR. +/+ Double infection by microscopy and single infection by PCR. +/- Single infection by microscopy and negative result by PCR.

	=	<>	-/+	+/+	?/+	?/+	+/+	+/-
examination	135	3	30	6	9	2	1	6
re-examination	183	0	5	2	0	1	1	0

