INFECTIOUS DISEASES

Diagnosis of Malaria by Polymerase Chain Reaction

IVÁN FERRER-RODRÍGUEZ, MS, MT; GUILLERMO J. VÁZQUEZ, MD; GLADYS J. CÓRDOVA, MD*; ADELFA E. SERRANO, PhD.

ABSTRACT. Malaria is no longer endemic in Puerto Rico, however, imported cases of the disease are occasionally reported to the Health Department of the Island. This is a report of a 45-year-old female patient who traveled to Kenya and Niger and was admitted to a San Juan area hospital with an 8 day history of daily chills and fever, myalgia, nausea and vomiting. Upon admission, peripheral blood displayed multiple intra-erythrocytic ring-shape trophozoites, highly suggestive of *Plasmodium falciparum*. The polymerase chain reaction was used as a complementary method for the detection of malaria parasites and confirmation of post-treatment parasite clearance. This report presents an imported case of malaria in Puerto Rico and showed the use of a molecular technique to diagnose *Plasmodium*. *Key words: Plasmodium falciparum, Imported malaria, Reemergent disease, Polymerase chain reaction.*

Malaria, an infectious condition caused by any of four protozoan parasites of the genus *Plasmodium*, is naturally transmitted to humans by the bite of an infected female *Anopheles* mosquito. In addition, the disease can be transmitted by the transfusion of infected blood (1), sharing of contaminated needles and by transplacental passage from an infected mother to the fetus (2).

Malaria is the world's most important parasitic infection with an estimate of 300-500 million cases and between 1.5 to 2.7 million deaths annually. The disease is endemic in 91 countries in tropical and subtropical regions and about 40% of the world's population is at risk (3). Malaria was a serious health problem in Puerto Rico during the first half of the 20th century, but later became the first tropical country in America to eradicate the disease and was certified as malaria-free by the World Health Organization in 1962 (4). However, more than 200 imported cases of malaria have been reported to the Health Department of Puerto Rico since eradication (5). Traditionally, the diagnosis of *Plasmodium sp.* in the previous cases was performed by blood smears, a simple technique that depends on the direct detection of parasites upon microscopic examination of Giemsa-stained slides. In the present report, a novel molecular diagnostic technique, the polymerase chain reaction (PCR), was used as a complementary method for the detection of malaria parasites and confirmation of post-treatment parasite clearance in a 45-years-old Puerto Rican female who had travelled to the African continent.

Materials and Methods

Microscopy. Peripheral blood samples were collected by venipucture for blood cell analysis. Thin blood smears were prepared at the time of blood collection or CBC processing upon initial patient evaluation, every eight hours for the next three days and daily thereafter. The slides were air-dried, stained with Giemsa and examined microscopically. Parasitemia was calculated by determining the number of infected erythrocytes per 100 red blood cells.

Polymerase chain reaction. Blood samples were collected for PCR analysis on days 3 and 10 after admission. For the PCR, 20 ml of blood were mixed with 200 ml of lysis buffer (50mM NaCl, 0.015% saponin, 1mM EDTA), centrifuged, and the supernatant decanted.

From the Department of Microbiology and Medical Zoology, University of Puerto Rico School of Medicine and the *Hospital Metropolitano, Rio Piedras, Puerto Rico.

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Address for correspondence: Adelfa E. Serrano, PhD. Department of Microbiology and Medical Zoology, University of Puerto Rico School of Medicine, PO Box 365067 San Juan PR 00936-5067 Tel. (787) 758-2525, ext 1313 Fax (787) 758-4808.

The samples were washed with two-hundred microliters of PCR buffer (70mM Tris, 20mM $(NH4)_2SO4$, 2.5mM $MgCl_2$, 1mM DTT, 0.1% Triton X-100, 50mg/ml BSA), transferred to 0.25ml vials containing PCR reaction mix (PCR buffer, 0.2mM deoxynucleotides, 50pmol oligonucleotide primers), and incubated for 10 min at 100°C. The enzyme, 1.25U Taq polymerase (Amplitaq™, Perkin-Elmer Cetus), was added to the 50µl reaction mix. The samples were amplified in a thermocycler (GeneAmp PCR System 2400, Perkin Elmer Cetus, Norwalk, CT) with an initial cycle of 60 sec at 94°C, followed by 39 cycles (30 sec. 80°C, 30 sec. 45°C, 30 sec. 72°C) and visualized by ethidium bromide staining of 2.0% agarose gels. Each experiment included a positive control containing 50ng of *P. falciparum* DNA and a negative control containing no target DNA.

Oligonucleotide primers. A set of *P. falciparum* specific oligonucleotide primers described by Tirasophon and Panyin (6), which generate a 206bp PCR product, were used in the study (5' GCTACATATGCTGTTGCCAG-AAC3'; 5' CTGGTACCATACATCCTACCAAC3').

Case History

A 45-years-old female was admitted to a private hospital of the San Juan area with a history of chills and fever, myalgia, nausea and vomiting of 8 days duration. The patient had returned twelve days earlier from a three-week tour of Kenya and Niger. She denied headaches, neurological changes, bleeding tendencies, urine discoloration or respiratory symptoms. Her vital signs were: temperature 103°C, blood pressure 90/40 mmHg, heart rate 100/min and respiratory rate of 22/min. She was acutely ill, alert and oriented. The only remarkable findings upon physical examination were a dehydrated oral mucosa and mild abdominal right upper quadrant tenderness. Pertinent admission laboratory examinations showed: hemoglobin of 12.6 grams; a white blood cell count of 6,400/mm³ (77% polymorphonuclear cells, 14% lymphocytes and 9% monocytes), platelet count of 73,000/mm³; a total bilirubin of 1.6 mg/dl (normal 0.2 to 1.3 mg/dl); alkaline phosphatase of 202 U/L (normal 45 to 122 U/L); lactic dehydrogenase of 1,159 U/L (normal 313 to 618 U/L); AST 130 U/L (normal 5 to 40 U/L); and ALT 168 U/L (normal 7 to 56 U/L). Arterial blood gases revealed a pH of 7.474, pCO_2 of 31 mmHg and pO_2 of 57.4 mmHg and a normal chest x ray. The peripheral blood smear demonstrated multiple intraerythrocytic ring-shaped trophozoites (approximately 50% parasitemia), highly suggestive of *Plasmodium falciparum*.

The patient was treated with quinine sulfate 600 mg orally three times/day, doxycycline 100 mg orally twice/day and clindamycin 900 mg intravenously every 8 hours, for a total of 10 days. In addition, careful hydratation and supplemental oxygen were administered. Her hospital course was remarkable, since she was asymptomatic by the third hospital day. Repeated malarial blood smears showed a decrease in the intraerythrocytic ring forms and by the 7th day they were reported as negative. Upon discharge, 12 days later, pertinent laboratory values showed: hemoglobin of 9.4 grams, white blood cell count of 4,800/mm³, platelet count of 506,000/mm³ and the lactic dehydrogenase was down to 864 U/L. Serum bilirubin, transaminases and other tests were within the normal range. She has remained afebrile, asymptomatic and with negative blood smears six months after discharged from the hospital.

Results

Microscopic examination of stained thin smears of peripheral blood revealed the presence of the typical ring-shaped trophozoites of *Plasmodium* (Fig. 1). Schizonts, gametocytes and multiple ring stages per cells were not observed. Initial parasitemia was higher than 50%. Subsequent examinations, following antimalarial therapy showed a gradual reduction in the number of parasites. After seven days of treatment, parasites were not detected by microscopic examination.

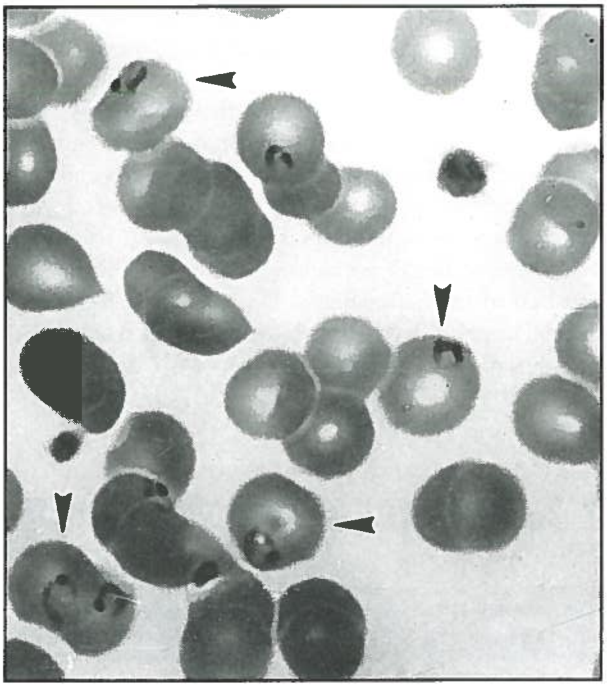

Figure 1. *P. falciparum* ring stages (arrows) detected during microscopic examination of a blood sample from the infected patient (Giemsa stain, thin smear, original magnification 1,000X).

The results of the PCR performed with the blood samples from the patient and the *P. falciparum* specific primers (6) showed a band of the expected size (approximately 206 bp, Fig. 2, lane 2). Ten days after the initial microscopic diagnosis the patient's samples were negative by PCR (Fig. 2, lane 3). The positive control using DNA from *P. falciparum* showed a band (206 bp) of the expected size (Fig. 2, lane 4). No band was observed in the negative control, in which no target DNA was included (Fig. 2, lane 5).

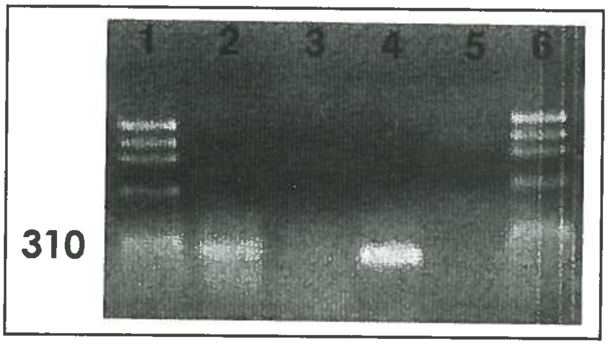

Figure 2. Agarose gel (2.0%) electrophoretic analysis of the PCR products. 1 & 6: DNA Standard Marker (φx174-HaeIII), 2: Positive PCR reaction from a blood sample three days after the initial microscopic diagnosis, 3: Negative PCR reaction from a blood sample ten days after the initial microscopic diagnosis, 4: Positive PCR reaction (206 bp band) using control DNA from *P. falciparum* and 5: Negative PCR reaction (no band) in the absence of target DNA. Note the expected diagnostic band of ~206bp in the patient's sample (lane 2). No amplification was observed in the patient's sample ten days post-treatment (lane 3), confirming parasites clearance.

To our knowledge, this is the first reported case of malaria in Puerto Rico, in which a molecular technique such as PCR has been used as a diagnostic tool. Three days after the initial microscopic diagnosis, a collected blood sample from the patient showed positive results by PCR (Fig. 2, lane 2). After therapy, a gradual reduction in the number of parasites in the blood was observed and by the 7[th] post-treatment day, Giemsa stained slides were reported as negative. Parasite clearance was further confirmed 10 days after the initial diagnosis by a negative PCR (Fig. 2, lane 3).

Table 1. Drugs used in the chemoprophylaxis of malaria.

Areas with chloroquine-sensitive *P. falciparum*	
Chloroquine phosphate (Aralen®)	300 mg base (500 mg salt) orally, once/week
Hydroxychloroquine sulfate (Plaquenil®)	310 mg base (400 mg salt) orally, once/week.
Areas with chloroquine-resistant *P. falciparum*	
Mefloquine (Lariam®)	228 mg base (250 mg salt) orally, once/week.
Doxycycline	100 mg orally, once/day.
Proguanil (not available in U.S.) (Paludrine®)	200 mg orally, once/day in combination with weekly chloroquine.
Prevention of relapses for *P. vivax* or *P. ovale*	
Primaquine	15 mg base (26.3 mg salt) orally, once/day for 14 days.

From CDC Travel Page, 1996 (30).

Discussion

The clinical history of the patient in this report included travel to Kenya and Niger, countries where chloroquine resistant *P. falciparum* is endemic. Chemoprophylaxis with chloroquine sulfate failed to protect the traveler and she developed the characteristic symptoms of malaria 4 days after returning home.

The condition of the patient improved rapidly after treatment with quinine sulfate, doxycycline and clindamycin. The clinical manifestations of the disease disappeared and no resistance to the therapeutic regimen was observed. The drugs used in the prophylaxis and treatment of uncomplicated malaria, as recommended by the Center for Disease Control are listed in Tables 1 and 2.

The use of molecular diagnostic techniques, such as the PCR, have significant advantages for diagnosing malaria (7-21). This PCR-based method has higher sensitivity (12, 13, 20, 22) and specificity than the traditional diagnostic method, based on the direct detection of parasites by microscopic examination of Giemsa stained slides. Furthermore, the PCR can directly detect parasites independently of the immunocompetence or previous clinical history of the patient and is capable of distinguishing between organisms that are morphologically similar (13, 20, 21). This method is able to detect a single *Plasmodium* parasite in a blood sample (11, 23) and only detect active infections (14).

Malaria is considered a reemergent disease as a consequence of the development of resistance by the parasite to anti-malarial drugs (24, 25). This resistant

Table 2. Drugs used for oral treatment of uncomplicated malaria in adults.

Acquired in chloroquine-sensitive areas	
Chloroquine phosphate	600 mg base (1,000 mg salt) followed by 300 mg base (500 mg salt) 6 hours later, then 300 mg base (500 mg salt) at 24 and 48 hours.
Acquired in chloroquine-resistance areas	
Drug of choice	
Quinine sulfate	650 mg every 8 hours for 3-7 days, plus
Tetracycline	250 mg 4 times/day for 7 days.
Alternatives	
Quinine sulfate	as above, plus
Pyrimethamine sulfadoxine (Fansidar®)	3 tablets at once on last day of quinine, or plus
Doxycycline	100 mg 2 times/day for 7 days, or plus
Clindamycin	900 mg 3 times/day for 3 days.
Mefloquine (Lariam®)	15 mg/kg salt, single dose, up to a maximum of 1,000-1,250 mg.
Halofantrine (Hallan®)	6 tablets (250 mg each), 3 doses of 2 tablets 6 hours apart.
Prevention of relapses for *P. vivax* or *P. ovale*	
Primaquine	15 mg base (26.3 mg salt) once/day for 14 days.

From Zucker and Campbell, 1993 (31) and Armitage, 1999 (32).

phenotype has spread rapidly with the increase in international travel and has introduced malaria to countries where the disease had been previously eradicated or to geographic areas where the disease was not endemic (26, 27, 28).

All travelers to malarious areas should be advised to start appropriate chemoprophylaxis before traveling, to continue its use while in malaria-endemic zones and for four weeks after leaving such areas (Table 1). The prophylaxis should be based on the pattern of malaria drug resistance present in the country of interest (29). It is advisable that travelers should take personal protection measures against the bites of mosquitoes such as insect repellents, mosquito nets, and clothes that cover most of the body.

The potential for an upward trend in the number of cases in Puerto Rico exists, reflecting the worldwide resurgence of malaria as a consequence of parasite drug resistance, increasing mosquito resistance to insecticides, the breakdown of malaria control campaigns, rapid population increase, immigration from overseas endemic areas, increased travel to malarious zones and war. Since the *Anopheles albimanus*, a highly effective vector of malaria, as well as other anopheline species capable of transmission are present in low levels in the Puerto Rico (Paul Reiter, CDC San Juan Laboratories, personal communication), the potential for transmission and spread is present in the Island.

Imported cases of malaria have been reported in Puerto Rico, almost every year since the disease was eradicated in 1962 (4, 5). The Puerto Rico Health Department should maintain a malaria surveillance program to provide appropriate advice to health professionals who identify and treat these cases. This malaria control strategy advocates a prompt and adequate diagnosis and treatment as essential tools to reduce the chances of mortality due to the disease. International travelers to malarious zones should be advised to practice appropriate prophylactic measures to reduce the risk of malaria infection.

This report presents a clinical case of imported malaria (from a chloroquine-resistant country) in Puerto Rico and demonstrates the feasibility of molecular diagnosis of malaria in the Island.

Resumen

La malaria no se considera una enfermedad endémica en Puerto Rico, sin embargo, casos importados de la enfermedad se reportan ocasionalmente al Departamento de Salud de la Isla. Informamos del caso de una paciente de 45 años de edad que viajó a Kenya y Niger y fue ingresada en un hospital privado del área de San Juan con historial de escalofríos, fiebre, mialgia, naúseas y vómitos durante 8 días de duración. Al momento de hospitalización, los exámenes de sangre mostraron múltiples trofozoitos intracelulares que sugerían *Plasmodium falciparum*. La reacción de polimerasa en cadena (PCR) fue utilizada como método complementario para la detección de parásitos y para confirmar la eliminación de parásitos con el tratamiento. Este reporte presenta un caso de malaria importada en Puerto Rico y muestra un diagnóstico de *P. falciparum* utilizando técnicas moleculares.

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