24

Submicroscopic Human Parasitic Infections

Fousseyni S. Touré Ndouo
Medical Parasitology Unit,
Centre International de Recherches Médicales de Franceville (CIRMF), Franceville
Gabon

1. Introduction

Polymerase chain reaction (PCR) amplification provides a powerful tool for parasite detection. This chapter examines the use of PCR to diagnose malaria in patients with low parasite densities (submicroscopic infections, SMI) and also occult loaiosis (OL: *Loa loa* infection without detectable circulating microfilaria on standard microscopy). It provides therefore the issue of management of these kinds of infections with regard to the eradication policy of such pathogens.

1.1 Classification

i. **Malaria:** Malaria is caused by *Plasmodium* parasites, of which there are about 200 species (Levine ND 1980). These protozoans belong to the *Apicomplexa* phylum, *Sporozoa* class and *Haemosporidae* subclass (Levine ND 1970). They are obligatory intracellular parasites. Two successive hosts, humans and mosquitoes (Culicidea and Anophelinea), are necessary for their life cycle. Four main species infect humans, namely *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. A fifth species, *P. knowlesi*, is currently spreading in south-east Asia and Oceania. This species derived from chimpanzees has caused more than 250 human cases of malaria in Malaysia but is still considered to be zoonotic (Figtree et al. 2010). *P. falciparum* causes most life-threatening infections.
Human is the intermediate host for malaria, wherein the asexual phase of the life cycle occurs. The sporozoïtes, inoculated by the infested female *Anopheles* mosquito, initiate this phase of the cycle from the liver, and continue within the red blood cells. From the mosquito bite, tens to few hundred invasive sporozoïtes are introduced into the skin. Following the intradermal drop, some sporozoïtes are destroyed locally by the immune cells, or enter into the lymphatic vessels, and some others can find blood circulation (Megumi L et al. 2007; Ashley M et al. 2008; Olivier S et al. 2008). The sporozoïtes that find peripheral blood circulation reach the liver within a few hours. It has been recently demonstrated that these sporozoïtes travel by a continuous sequence of stick-and-slip motility, using the thrombospondin-related anonymous protein (TRAP) family and an actin–myosin motor (Baum J et al. 2006; Megumi L et al. 2007; Münter S et al. 2009). The sporozoites migrate into hepatocytes and then grow within parasitophorous vacuoles and develop to the schizont stage which releases merozoites (Jones MK et al. 2006; Kebaier C et al. 2009). The entire pre-eryhrocytic phase lasts about 5–16 days depending

on the parasite species (5-6 days for *P. falciparum*, 8 days for *P. vivax,* 9 days for *P. ovale,* 13 days for *P. malariae* and 8-9 days for *P. knowlesi.* The pre-erythrocytic phase remains a "silent" phase, with little pathology and no symptoms, as only a few hepatocytes are affected (Ashley M et al. 2008). This phase is a single cycle, contrasting to the next, erythrocytic stage, which occurs repeatedly.

ii. **Loaiosis:** Filariasis are typically chronic tropical diseases caused by nematodes of the *Filariidae* family, transmitted by flies or mosquitoes. Eight species are currently known to infect humans, namely *Wuchereria bancrofti*, *Brugia malayi, B. timoni, Onchocerca volvulus*, *Loa loa*, *Mansonella perstans*, *M. ozzardi*, and *M. streptocerca*. Three groups of filariasis have been distinguished on the basis of their human target tissues: lymphatic filariasis (wuchereriasis and brugiasis); cutaneous dermal filariasis (loaiosis, onchocerciasis, and *streptocerca* mansonelliasis) and serous filariasis (*perstans* and *ozzardi* mansonelliasis) (Gentilini 1982). The vectors are blood-sucking flies and female mosquitoes. The microfilarial eggs or embryos are ingested by the vector when it bites an infected human. These microfilariae become infective stage L3 larvae after two successive mutes within the vector, and are transmitted to a new human host through a new blood meal or bite.

More than 3.3 billion people are exposed to filariosis, and an estimated 300 million people are infected. Loaiosis occurs in Africa, brugiaioses in South Asia, wuchereriasis in Africa and Asia, onchocerciasis in Africa, Central and South America and Asia (Yemen), *perstans* mansonelliasis in Africa and Central and South America, *ozzardi* mansonelliasis in Central and South America, and *streptocerca* mansonellaiosis in Africa.

L. loa infection (loaiosis) was initially described in 1770 by Mongin, in a female slave originating from West Africa and living on Saint Domingue island. Guyon et al. found the same worm in Gabon (Central Africa) in 1864. *L. loa* was first described in detail by Brumpt *et al*. in 1904, and then by Connors *et al.* in 1976. Although discovered in the Antilles, *L. loa* is restricted to Africa (Gentilini 1982). The adult worms live under the skin for about 15 years (Gentilini et al. 1982). The tabanides responsible for *L. loa* transmission are primarily *Chrysops dimidiata* and *silacea*, two forest species often present in the same hearth. Only the females are hematophagous, and they have diurnal activity.

1.2 Pathogenesis

i. **Malaria:** *P. falciparum* is responsible for most complicated forms of malaria and causes about 800 000 deaths a year, mostly among children in sub-Saharan African countries (WHO 2009). Malaria symptoms generally occur in three phases. After an incubation period of 7 to 10 days, symptoms begin with fever, aches and digestive disorders (febrile stomach upset). Then, when schizont rupture becomes synchronous, patients enter the feverish reviviscent schizogonic phase (periodic fever) of uncomplicated malaria. This phase is characterized by fevers typically appearing every 24 hours (third fever in infection by *P. vivax* or *ovalae*, every 48 hours, quartan fever in infection by *P. malariae* or *P. falciparum*), accompanied by a triad of symptoms: shivers, fever and sweating. Destruction of parasitized red blood cells leads to the release of malarial toxins and to TNF alpha production. The third phase, mainly seen with *P. falciparum*, corresponds to severe malaria (pernicious access), which sometimes occurs rapidly after infection. Clinical and biological signs are used to classify malaria (WHO 2000 gravity

criteria, Imbert et al 2002). The reasons why some non immune individuals infected by *P. falciparum* develop severe malaria and die, while others have only uncomplicated malaria or remain asymptomatic, remain unclear (Marsh et al 1988). Severe anemia and cerebral malaria are responsible for most of the morbidity and mortality related to this disease in children. Despite abundant research, the pathophysiological mechanisms underlying severe forms are poorly understood. Several studies have implicated sequestration of *P. falciparum*-parasitized red blood cells (PRBC) in the lungs and brain (Taylor et al. 2001). This sequestration is characterized by PRBC adhesion (or cytoadherence), agglutination and rosetting. Cytoadherence of PRBC to host endothelial cells (EC) in brain and lung capillaries can obstruct the microvasculature, a phenomenon accompanied by changes in the T cell repertoire and by cytokine production (Mazier et al. 2000). This adherence is modulated by platelets (Brown et al. 2000) and is mediated by EC receptors such as CD36, intracellular adhesion molecule 1 (ICAM1), vascular cellular adhesion molecule 1 (VCAM1), CD31, integrins and hyaluronic acid (Hunt et al. 2003). PRBC adhesion can induce over-expression of inflammatory cytokines (Mazier et al. 2000) and EC apoptosis (Pino et al. 2003). Approximately 20% of *P. falciparum* isolates from Franceville, Gabon (Central Africa), were show to induce human lung endothelial cell (HLEC) apoptosis by cytoadherence (Touré et al. 2008). In addition, apoptogenic isolates were more frequent in children with neurological signs (prostration or coma), supporting the hypothesis that PRBC-mediated EC apoptosis could amplify blood-brain barrier disruption and dysfunction (Combes et al. 2005; Bisser et al. 2006). Whole transcriptome analysis revealed that 59 genes were more intensely transcribed in apoptogenic strains than in non apoptogenic strains (Siau et al. 2007). Knock-down of 8 of these genes by double-strand RNA interference significantly reduced the apoptogenic response in 5 genes (PF07_0032, PF10255, PFI0130c, PFD0875c, and MAL13P1.206). These five genes are known as *Plasmodium* apoptosis-linked pathogenicity factors (PALPF).

ii. **Loaiosis:** Loaiosis is characterized by calabar oedema (swelling) and conjunctivitis due to ocular passage of adult worms. Calabar oedema is transient and located on the face, limbs and back of the hands and fingers. *L. loa* is also called the "African eye worm". Meningoencephalic complications are an adverse effect of diethylcarbamazine treatment for hypermicrofilaremia. Other complications such as nephropathies, endocarditis, retinopathies, neuropathies and pneumonitis have been reported (Schofield et al. 1955; Hulin et al. 1994). Symptoms are more frequent in expatriates. Immunologically, loaiosis is characterized by hypergammaglobulinemia, hypereosinophilia and high IgE levels responsible for allergic symptoms. In endemic areas, loaiosis is the third reason for medical consultations in rural settings, although many microfilaremic subjects are asymptomatic. Occult loaiosis (amicrofilaremic infection) is defined as infection by the adult worm without peripheral microfilaremia on standard microscopy. Amicrofilaremic status is common among autochthonous residents and may be due to sequestration of microfilaria or to their massive destruction by the immune system, or to the presence of sterile adult worms. This form of infection is the most common in endemic areas. Other amicrofilaremic subjects are thought to be resistant. There is currently no way of discriminating between these two amicrofilaremic subgroups in the absence of (transient) ocular passage of adult worms.

1.3 Diagnostic challenges

i. **Malaria:** Light microscopy of blood smears remains the standard method for *Plasmodium* detection, both for clinical diagnosis and epidemiological surveys (Okell LC et al. 2009). However, sensitivity depends on parasite density in blood. In patients with low parasitemia, mixed infections, antimalarial treatment or chronic infection, microscopic diagnosis requires painstaking examination by an experienced technician. Low-density infections that cannot be detected by conventional microscopy are termed submicroscopic infections (SMI). *Plasmodium* species identification is mainly based on microscopic morphological characteristics but this is not entirely reliable (*Plasmodium vivax* resembles *P. ovale*). In addition, parasite morphology can be altered by drug treatment and/or sample storage conditions.

ii. **Loaiosis:** Human loaiosis differs from other filariasis by the fact that most patients have "occult" infection, with no circulating microfilaria. This peripheral amicrofilaremia can be due to microfilaria destruction by the immune system, and/or to their sequestration. These subjects cannot be diagnosed by microscopy and consequently go untreated, constituting a parasite reservoir. Before 1997, *L. loa* diagnosis was still based on microscopic examination and the prevalence was therefore underestimated. In contrast, because of cross-reactions, serological tests, and especially those based on total IgG detection, tend to overestimate prevalence. The existence of many cases of occult but symptomatic infection among residents in endemic areas implies the need for specific and sensitive detection.

2. PCR-based diagnosis of malaria

In 1993 a PCR method targeting the small subunit of the ribosomal RNA (SSUrRNA) gene was developed for use as an alternative to microscopy for detecting the four main *Plasmodium* species (Snounou et al. 1993, 1994, 1995). Nested PCR was used for its high sensitivity and specificity (Snounou et al. 1993). However, the nested reaction requires five separate PCR reactions and is therefore time-consuming, expensive and not always feasible in developing world laboratories. Several variants of this nested PCR method, such as semi-nested multiplex and one-tube multiplex have been developed (Mixon-Hayden T et al. 2010). In 1998 Jarra and Snounou showed that *Plasmodium* DNA is cleared very quickly from the bloodstream and that positive PCR amplification is usually associated with the presence of viable parasites. PCR positivity therefore indicates active *Plasmodium* infection. Since 1997, several PCR methods targeting other *P. falciparum* genes have been developed (Cheng et al. 1997; Filisetti et al. 2002). Their sensitivity has been estimated at 71%, 83% and 100% for the *MSP-2, SSUrRNA* and *STEVOR* genes, respectively (Oyedeji et al. 2007).

Real-time PCR has been reported to be able to improve parasite detection. Compared to *SSUrRNA* nested PCR, the real-time assay had a sensitivity of 99.5% and specificity of 100% for the diagnosis of malaria (Farcas GA et al. 2004). The real-time PCR method, specific for all *Plasmodium* species, avoids post-amplification sample handling and electrophoresis, and the result can be ready within 45 min (Farcas GA et al. 2004). This method would be useful for monitoring antimalarial drug efficacy, especially in areas of drug resistance (Lee MA et al. 2002).

More recently, it has been shown that dot18S (18SrRNA gene) and CYTB, two new molecular methods, are highly sensitive and allow high-throughput scaling up for many

hundred samples (Steenkeste N et al. 2009). The CYTB is a nested PCR based on Plasmodium cytochrome b gene followed by species detection using SNP (single nucleotide polymorphism) analysis. The usefulness of these methods in detecting malaria has been demonstrated especially in low endemic areas.

2.1 Materials and methods

a. **Blood sampling**
Samples must be collected in sterile tubes. For example, peripheral blood is collected in tubes containing an anticoagulant such as EDTA. However, some anticoagulants, such as heparin, inhibit the action of Taq DNA polymerase and should thus be avoided. Blood samples can also be collected in the form of drops on calibrated pre-punched paper disks (Serobuvard, LDA 22H, Zoopole, Ploufragan, France) (Ouwe-Missi-Oukem-Boyer et al. 2005).

b. **Microscopy**
Thick and thin peripheral blood films were stained with Giemsa and examined by microscope. For microscope positive samples, the parasite load is expressed as the number of asexual forms of *P. falciparum*/µL of blood, assuming an average leukocyte count of 8000/µL.

c. DNA template preparation
There are many useful techniques for DNA template processing. Plasmodial DNA extraction involves erythrocyte lysis and proteinase K digestion to prevent PCR inhibition.

i. DNeasy^{R} Blood & Tissue Kit: Whole blood (200 µl) is used for DNA extraction with the DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany). Briefly, DNA extraction is carried out as follows. To a 1.5-ml tube containing 200 µl of while blood are added 20 µl of proteinase K solution and 200 µl of AL buffer (a detergent included in the kit). The mixture is pulse vortexed for 15 seconds and incubated for 15 minutes at 56°C. Two hundred microliters of cold ethanol is then added and the mixture is vortexed for 15 seconds. The mixture is transferred to a mini-column assembled on a 2-ml tube and centrifuged for 1 min at 8000 rpm. After centrifugation the 2-ml tube is discarded OK. The mini-column is recovered and placed on a new 2-ml tube. The mini-column is then washed with 500 µL of AW1 buffer (available in the kit) by centrifugation at 8000 rpm for 1 min. This washing step is repeated with another 500 µL of AW2 buffer, followed by centrifugation for 3 min at 14 000 rpm. The 2-ml tube is again discarded.
The mini-column is placed on a 1.5-ml tube and 60 µl of AE elution buffer is added. This unit is left at room temperature for 10 min and then centrifuged for 1 min at 8000 rpm. The DNA is then recovered in the 1.5-ml tube and immediately used as a template or stored at 20°C.

ii. Dried blood-spot method (DBS)**:** DNA templates are extracted as described by Plowe CV et al in 1995. The dried blood spot is placed in 1 ml of phosphate buffered saline (PBS) containing 0.5% saponin and is incubated overnight at 4°C. The resulting brown solution is replaced with 1 ml of PBS and incubated for an additional 15-30 minutes at 4°C. Then, 200 µl of 5% Chelex 100 (Bio-Rad Laboratories, CA) is placed in clean tubes and heated to 100°C in a water bath. The disks are removed from the PBS and placed in the preheated 5% Chelex 100,

vortexed at high speed for 30 seconds and placed in a water bath at 100°C for 10 minutes with gentle agitation. The samples are then centrifuged at 10 000 *g* for 2 minutes, and the supernatant is removed and centrifuged as before. The supernatant is then collected in a clean tube and immediately used for PCR or stored at 20°C until use.

DNA can be also extracted from dried blood spots with several other methods, such as the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany).

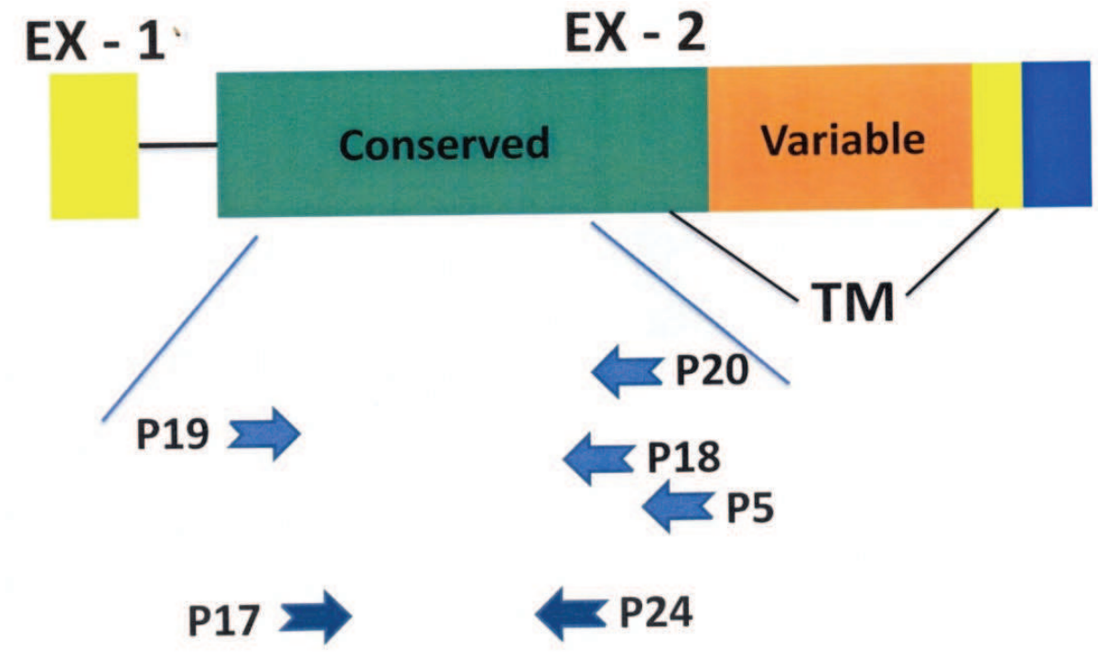
2.2 *P. falciparum* DNA amplification and detection

i. SSUrRNA gene amplification

Two microliters of DNA extract is amplified in a final volume of 25 µl containing 2.5 µl of 10X reaction buffer, 100 µM each dNTP (dATP, dGTP, dTTP, and dCTP), 0.5 pM each primer (rPLU5/rPLU6 (rPLU5 5'-CCT GTT GTT GCC TTA AAC TTC-3' and rPLU6 5'-TTA AAA TTG TTG CAG TTA AAA CG-3') for the primary reaction, and rFAL1/rFAL2 (rFAL 1 5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3' and rFAL 2 5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3') for the nested reaction) and 0.75 units of Taq DNA polymerase (QIAGEN, Hilden, Germany). The primer sequences (Table 1) are based on SSUrRNA sequences described elsewhere (Snounou et al. 1993). The PCR program is as follows: denaturation at 95°C for 5 min followed by 25 cycles (30 cycles in nested PCR) at 94°C for 1 min, 60°C for 2 min and 72°C for 2 min, with a final extension step of 5 min at 72°C.

ii. STEVOR gene amplification

The first round of amplification is performed with a reaction mix of 50 µl containing 5.0 µl of 10X reaction buffer, 200 µM each dNTP (dATP, dGTP, dTTP, and dCTP), 1.25 units of Taq



Schema 1. Schematic representation of the STEVOR PCR methodology (CHENG et al 1997).

DNA polymerase, 0.4 pM each primer (P5, P18, P19 and P20) (P5 5'-GGG AAT TCT TTA TTT GAT GAA GAT G-3', P18 5'-TTT CA(C/T) CAC CAA ACA TTT CTT-3', P19 5'-AAT CCA CAT TAT CAC AAT GA-3', P20 5'-CCG ATT TTA ACA TAA TAT GA-3') and 5 µl of DNA template. The PCR program is as follows: denaturation at 93°C for 3 min followed by 25 cycles of 30 s at 93°C, 50 s at 50°C and 30 s at 72°C, with a final extension step of 3 min at 72°C. Two microliters of the first-round PCR product is used for the second round of amplification, with a reaction mixture of 50 µl containing 5.0 µl of 10X reaction buffer, 200 µM each dNTP, 1.25 units of Taq DNA polymerase and 0.4 pM each primer (P17 and P24) (P17 5'-ACA TTA TCA TAA TGA (C/T) CC AGA ACT-3', P24 5'-GTT TGC AAT AAT TCT TTT TCT AGC-3'). The PCR conditions for the nested reaction are as follows: denaturation at 93°C for 3 s, followed by 25 cycles of 30 s at 93°C, 50 s at 55°C and 30 s at 72°C, with a final extension step of 3 min at 72°C.

iii. Detection procedures

Analysis of PCR products: After amplification, 10 µl of each PCR product is mixed with 1 µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% w/v sucrose in water) and analyzed by electrophoresis on 1.5% agarose gel. The gel is stained with ethidium bromide or FluoProbes Gel Red (Interchim Montlucon, France) and the DNA is visualized and photographed under ultraviolet light.

***Plasmodium* SSUrRNA gene:**

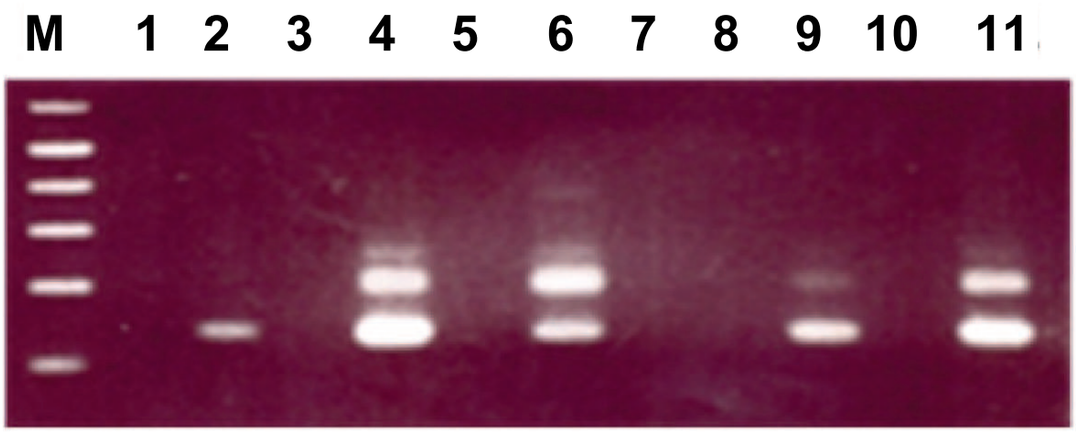


Fig. 1. Detection and speciation of *Plasmodium* by nested PCR using genus-specific primers and 1.5% agarose gel electrophoresis. Lanes 1 and 8: PCR-negative controls; lane 2: an individual with submicroscopic infection by *P. malariae* (size: 144 base pairs); lanes 3, 5, 7 and 10: PCR-negative individuals; lanes 4, 6, 9 and 11: individuals with submicroscopic co-infection with *P. falciparum* (size: 205 bp) and *P. malariae*. Lane M represents the DNA molecular weight marker (100 bp).

***P. falciparum* STEVOR gene**

Theoretically, three specific bands between 189-700 base pairs are generated using nested primers. We obtained a specific band of 250 bp for all *P. falciparum* isolates tested in Franceville, southeastern Gabon.

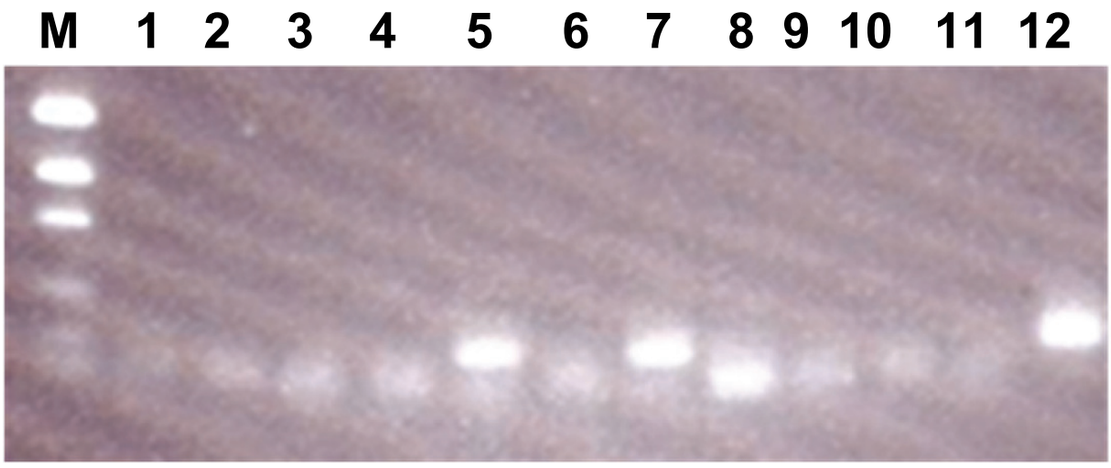


Fig. 2. Detection of the *Plasmodium* STEVOR gene by nested PCR using specific primers and 1.5% agarose gel electrophoresis. Lanes 1 and 10: PCR-negative controls; lanes 2, 3, 4, 6, 8, 9 and 11: PCR-negative samples; lanes 5 and 7: PCR-positive samples; lane 12: PCR-positive control; lane M: DNA molecular weight marker (123 bp).

3. PCR based diagnosis of *Loa loa*

In 1997, a PCR method (15r3-PCR) was developed to detect the repeat 3 region of the gene encoding the *L. loa* polyprotein in blood samples (Touré et al. 1997a, 1997b). Amicrofilaremic status is generally due to massive destruction of microfilaria, releasing parasite DNA into the bloodstream. These molecules may exist free in plasma, or be associated with cell-surface proteins, or even be contained in phagocytic cells. In addition, the adult worms can release DNA when they produce nonviable eggs or when they die after immune attack. The quantity of DNA released, whether from eggs, microfilaria or adult worms, is related to the parasite load of adult worms. The 15r3-PCR assay had a sensitivity of 95% with respect to detection of ocular passage of *L. loa* adult worms, and 100% compared to detection of microfilaremia.

3.1 Materials and methods

a. Blood sampling
   As previously mentioned blood samples must be collected by venipuncture into Vacutainer tubes containing an adequate anticoagulant such as EDTA.

b. Leukoconcentration
   Clinically, *L. loa* infection is diagnosed when migration of adult worms under the conjunctiva and/or skin is observed, or when a patient presents with classical symptoms. Diagnosis is classically based on standard microscopy. Microfilariae are the blood stage of *L. loa*. One milliliter of each blood sample is added to a 15-ml tube containing 9 ml of phosphate buffered saline (PBS), in duplicate. The mixture is treated with 600 µl of 2% saponin at room temperature for 15 min to lyse red blood cells, followed by centrifugation at 1000 *g* for 15 minutes at 4°C. The supernatant is discarded and the pellet is examined microscopically for microfilariae. The distinction between *L.*

loa and *M. perstans* microfilariae is based on size, motility, and by the presence of a sheath (*L. loa)*.

Thick smears can be also prepared with venous blood and stained with Giemsa or hematoxylin-eosin to detect microfilariae. The QBC (Quantitative Coating Buffer) method is also used to detect *L. loa* microfilariae.

For microscopic detection of *L. loa* microfilariae, blood samples must be collected during the day, given the diurnal periodicity of human loaiosis.

c. Whole blood lysate processing

Whole blood (100 µl) is mixed with 500 µl of TE buffer (10 mM Tris pH 8; 0.1 mM EDTA pH 8) and spun at 10 000 *g* twice for 2 min, discarding the supernatant at each step. The pellet is resuspended in 500 µl of sucrose buffer (10 mM Tris pH 7.6, 5 mM $MgCl_2$ 1 M sucrose and 1% Triton X 100) and spun at 10 000 *g* twice for 2 min. After the final wash, the supernatant is discarded and the pellet is resuspended with 200 µl of prewarmed (56°C) proteinase K buffer (containing 20 mM Tris pH 8, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µg/ml proteinase K and 0.5% Tween20), incubated at 56°C for two hours, then held at 90°C for 10 min. The DNA can be stored at 4°C for several days or at -20°C until required.

d. *L. loa* 15r3 gene amplification and detection

Primers corresponding to the 5' and 3' ends of the repeat 3 sequence of the gene coding for *L. loa* 15 kDa allergenic polyprotein are used. Primary amplification is done with a reaction mixture of 50 µl containing 2 µl of blood lysate, 1X PCR buffer (supplied by the manufacturer: 200 mM Tris-HCl pH 8.7, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton x100, 1 mg/ml bovine serum albumin), 200 µM each dATP, dCTP, dGTP and dTTP, 1 µM each primer (15r3-1: 5'-AAT-CAG-GCA-AAT-AAT-GGC-ACA-AAA-3', 15r3-2: 5'-GCG-TTT-TCT-TCT-CAC-CAG-CTG-TCT-3') and 1 unit of DNA polymerase. Amplification is performed with a Perkin Elmer thermal cycler for 40 cycles: 94°C for 1 min (denaturation), 65°C for 1 min (annealing) and 72°C for 2 min

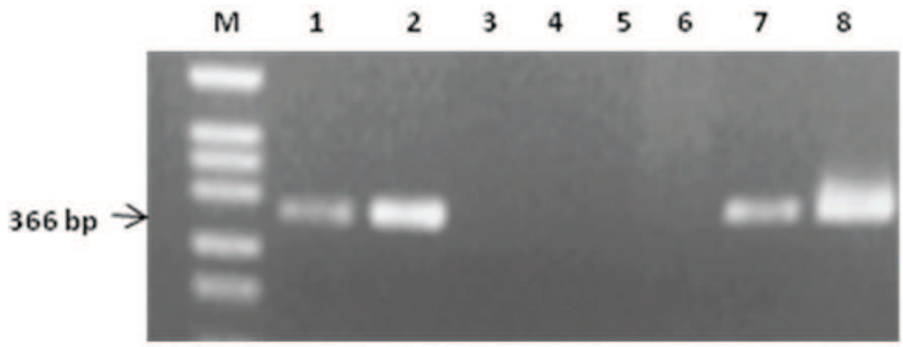


Fig. 3. Representative 1.5% agarose gel electrophoresis patterns of nested 15r3 PCR products. Lanes 1, 2 and 7: *L. loa* amicrofilaremic individuals (AMF) positive by PCR. Lanes 4 and 5: individuals negative by PCR. Lane 8: an individual with 100 *L. loa* microfilariae per ml and positive by PCR. Lanes 3 and 6: PCR-negative controls (no template); 5 µl of each nested PCR product was applied to each lane and revealed using UV transillumination after ethidium bromide staining. A fragment of 366 bp was observed with positive samples. Lane M: DNA molecular weight marker VI (Boehringer).

(extension), preceded by a ”hot start” cycle at 96°C for 10 min, 80°C for 5 min and 94°C for 30 s. One microliter of product from the first-round amplification is used for a second round in the above conditions for 30 cycles. The following primers are used: 15r3-3: 5’GGC ACA AAA CAC TGC AGC AGT CCT3’, and 15r3-4: 5’CAG CTG TCT CAA ATC GAA GAT TCT 3.’

4. Submicroscopic infection and disease management and control

i. Malaria:

The global strategy for malaria control is based on prevention, early diagnosis and prompt treatment. The detection limit of routine microscopy has been estimated to be about 100 parasites/milliliter, whereas PCR can detect as little as 0.01 parasite /micro liter (Mockenhaupt FP et al. 2002). Submicroscopic infection (SMI) including submicroscopic gametocytes is common in both symptomatic and asymptomatic individuals with malaria. A systematic review and analysis of field data carried out by Okell LC et al. in 2009 showed that the prevalence of *P. falciparum* was twice as high with PCR as with microscopy. In a village in Dienga, southeastern Gabon, PCR was performed on blood samples from asymptomatic individuals negative by microscopy: the prevalence of SMI (PCR positivity) was 13.7% by PCR and 7.2% by microscopy (Touré et al. 2006). A study carried out by Bouyou-Akotet et al. in 2010 in Libreville (capital of Gabon) showed an 18.2% prevalence of SMI in pregnant women. Recently, SMI was detected in 18% of symptomatic individuals in Franceville, southeastern Gabon, whereas the microscopic prevalence was 23% (author’s personal data). It has been estimated that as many as 88% of infections remain undetectable by microscopy in low-transmission areas, where the PCR prevalence is generally under 10% (Okell LC et al. 2009). Thus, a high rate of SMI could undermine disease control programs. In endemic areas, it has been shown that *P. falciparum* SMI contributes to acute disease (Rogier C et al. 1996), and to malaria-associated anemia and inflammation (Mockenhaupt FP et al. 2002). It has also been shown that cerebral malaria is frequently associated with SMI in semi-immune individuals (Giha HA et al. 2005). Finally, Bouyou-Akotet et al. 2010 have demonstrated that SMI during pregnancy is associated with low birth weight, especially in primagravidae. As parasite resistance to antimalarial drugs is currently widespread and increasing, it is very important to identify resistant parasites in patients with SMI. Two major genes have been implicated in *P. falciparum* resistance to quinoline, namely *Pfcrt* (*P. falciparum* chloroquine resistance transporter) and *Pfmdr1* (*P. falciparum* multidrug resistance gene 1). Single-nucleotide polymorphisms (SNPs) in these genes are associated with resistance both *in vitro* and *in vivo* (Wongsrichanalai et al. 2002). Therefore, *P. falciparum* drug resistance is linked to particular parasite genotypes (Duraisingh et al. 1997). *P. falciparum* infection is generally polyclonal, and may thus involve both drug-sensitive and resistant genotypes. SMI detection can be used to evaluate the therapeutic effectiveness of anti-malarial drugs during mass treatments and preclinical trials.

SMI individuals are capable of infecting mosquitoes and contributing to human transmission (Coleman RE et al. 2004), mainly in areas of seasonal transmission (Nwakanma D et al. 2008). Microscopy fails to detect the parasite in 49.2% of all malaria cases and in 91.3% of gametocytemic individuals (Okell LC et al. 2009). Individuals whose blood smears are negative for gametocytes (submicroscopic gametocyte) are equally able to transmit the infection to mosquitoes as slide-positive individuals (Coleman RE et al. 2004). Thus, the SMI

gametocyte reservoir may sustain malaria transmission despite efforts to fight malaria in endemic areas (Karl S et al. 2011). The prevalence of SMI, including submicroscopic gametocytes, must be assessed and taken into account in malaria control programs (Okell LC et al. 2009, Karl S et al. 2011).

Only patients with positive blood smears and/or rapid diagnostic tests (RDT) are routinely treated, while the treatment of patients negative by both methods depends on clinical signs and the physician's appreciation. These patients, including those with SMI, may represent more than 10% of infected individuals. In Gabon, SMI currently tends to be more frequent than microscopic infection, possibly due to better preventive policies and/or case management (Bouyou-Akotet et al. 2010). Treatment of all infected subjects, including those with SMI and submicroscopic gametocytes, would reduce the community parasite burden. Indeed, it has been shown that intermittent preventive treatment can reduce the prevalence and genetic diversity of *P. falciparum* malaria (Liljander A et al. 2010).

ii. *Loa loa*

Human loaiosis differs from other filariasis by the fact that most infected individuals do not have blood microfilariae detectable by standard microscopy. Since the first description of this filariasis, many epidemiologists have found a low prevalence of microfilaria despite local vector abundance. The notion that most patients clear their microfilaremia but continue to have (occult) infection is primarily based on the observation of adult worms during eye passage. The assumption that endemic resistant subjects also may exist (subjects able to completely eliminate *L. loa* infection) is still based on the same observations. Only a sensitive diagnostic test can confirm these assumptions. Our results have shown that 15r3-PCR is suitable for discriminating among endemic groups (microfilaremics, occult infected individuals (occults) and resistant subjects), as the results should be positive in the first two groups and negative in the last. Indeed, two-thirds of infected individuals in southeastern Gabon have occult loaiosis (OL) Touré et al. (1998, 1999a). This needs to be shown in a longitudinal study, however, as *L. loa* infection is characterized by its relative stability in humans and mandrills, the adult worm having a lifespan of about 15 years (Gentillini 1982, Pinder 1994).

This implies that the prevalence of loaiosis would be underestimated by microscopy. If *L. loa* DNA detection is a marker of active infection, all subjects positive by PCR should be treated. This would not have a major impact on health at the individual level but could reduce the parasite burden in the community, in turn reducing the intensity of transmission and resulting in public health benefits.

Studies of resistant individuals may provide interesting immunological information. Marked differences in humoral and cellular immune responses have already been noted between microfilaremic and amicrofilaremic patients (Pinder 1988, Akué 1997, Baize et al. 1997), as well as in the mandrill model (Leroy 1997). However, lacking a reliable method for diagnosing occult infection, it is not known if this difference is due to immunity directed against adult worms or against microfilaria. The identification of endemic groups ("microfilaremics", "occults" and "resistants") by 15r3-PCR method should allow immunological studies to be carried out with sera and cells from each endemic group, using antigens of each developmental stage of *L. loa*, and particularly infective larvae and adult worms. Such studies could help to identify possible cellular or humoral markers involved in

resistance to infection, as well as the underlying mechanisms, including host genetic factors. These studies would open the way to investigations of the underlying molecular mechanisms.

In addition, the detection of OL by PCR will allow precise evaluation of filaricide effectiveness during mass treatment, and also that of new drugs in animal models. Pinder et al. showed in 1994 that experimental mandrill infection (*Mandrillus sphinx*) by human *L. loa* isolates led to the same parasitologic characteristics as the natural human infection. Thus, mandrills with occult infection (absence of microfilarae but presence of adult worms, as shown by 15r3-PCR positivity; Touré et al. 1998) can be used to evaluate macrofilaricidal drugs. It has been demonstrated that the 15-kDa polyprotein is conserved within human and simian *L. loa* (Touré et al. 1999b). L.15r3-PCR also detects simian occult *L. loa* and could be used to identify infected animals before their inclusion in preclinical trials.

Finally, serological tests using purified recombinant antigens or peptides offer much better specificity than those using crude antigens. When these antigens become available for loaiosis, immunoenzymatic methods like IgG4 ELISA will reach acceptable specificity. Comparison of ELISA and PCR results sould show whether or not specific IgG4 antibodies are markers of active *L. loa* infection.

5. Conclusion

The global strategy of eliminating the parasitic diseases especially malaria and filariasis is mainly based on prevention, early diagnosis and prompt treatment. However, most decisions still rely on microscopy diagnosis which is not always adapted in detecting all infections. Indeed, the success of any intervention depends of the effectiveness of tools and methods especially those allowing proper detection of parasites. PCR offers an exciting opportunity to diagnose submicroscopic malaria infections and occult loaiosis which may constitute a hidden reservoir of disease transmission. The detection of such infections would allow the accurate management of all cases necessary to progress from disease control to elimination.

6. References

[1] Levine ND. et al. A newly revised classification of the protozoa (1980). *Protozool*. 27, 37-58.

[2] Levine ND. Protozoan parasites of nonhuman primates as zoonotic agents (1970). *Lab Anim. Care* 20,377-82.

[3] Figtree M, Lee R, Bain L, Kennedy T, Mackertich S, Urban M, Cheng Q, Hudson BJ. Plasmodium knowlesi in human, Indonesian Borneo (2010). *Emerg Infect Dis*. Apr;16 (4):672-4.

[4] Megumi Y L, Alida Coppi, Georges Snounou, Photini Sinnis. Plasmodium sporozoites trickle out of the injection site. *Cell Microbiol*. 1 May 2007;9(5):1215–1222.

[5] Ashley M. Vaughan, Ahmed S. I. Aly, Stefan H. I. Kappe. Malaria parasite pre-erythrocytic stage infection: Gliding and Hiding. *Cell Host Microbe*. 11 September 2008;4(3):209–218.

[6] Olivier Silvie, Maria M Mota, Kai Matuschewski, Miguel Prudêncio. Interactions of the malaria parasite and its mammalian host. *Current Opinion in Microbiology* 2008;11:352–359.

[7] Jake Baum, Dave Richard, Julie Heale et al. A Conserved Molecular Motor Drives Cell Invasion and Gliding Motility across Malaria Life Cycle Stages and Other Apicomplexan Parasites. *The Journal of Biological Chemistry*. February 2006;281:5197-5208.

[8] Sylvia Münter, Benedikt Sabass, Christine Selhuber-Unke et al. Plasmodium Sporozoite Motility Is Modulated by the Turnover of Discrete Adhesion Sites *Cell Host & Microbe.* December 2009;6 (17):551-562.

[9] Malcolm K Jones, Michael F Good. Malaria parasites up close. *Nature Medicine* 2006; 12:170-171.

[10] Kebaier C, Voza T, Vanderberg J. Kinetics of Mosquito-Injected Plasmodium Sporozoites in Mice: Fewer Sporozoites Are Injected into Sporozoite-Immunized Mice. *PLoS Pathog* 2009; 5 (4):e1000399.

[11] Gentillini M., Duflo B. (1982). Filarioses. Médecine Tropicale- Paris: Flammarion. 173-195.

[12] Mogin Observations sur un ver trouvé dans la conjonctive à maribou. île Saint Domingue (1770). Jour. Med. Chirurgie Pharm. etc (Paris). 32 : 338-339. Guyon J (1964). Sur un nouveau cas de filaire, sous conjonctive, ou Filaria occuli des auteurs, observe au Gabon (Côte occidentale d'Afrique). *C. R. Séances Acad. Sci*. 59 : 743-748.

[13] Guyon J. (1864). Sur un nouveau cas de filaire, sous conjonctive, ou Filaria occuli des auteurs, observe au Gabon (Côte occidentale d''frique). *C. R. Séances Acad. Sci*. 59 : 743-748.

[14] Brumpt E. (1904). Les filarioses humaines en Afrique. *Compte Rendu de la Société de Biologie*, 56 : 758-760.

[15] Connor DH, Neafie RC, Meyers WM. (1976). Loiasis : Pathology of tropical and extra-ordinary diseases. Washington: Armed Forces Institute of Pathology. 356-359.

[16] World Health Organization (2009). WHO World Malaria report 2009.

[17] World Health Organization. Severe falciparum malaria (2000).*Trans R Soc Trop Med Hyg*; 94 (Suppl. 1): S1–S90.

[18] Imbert P and Gendrel D (2002). [Malaria treatment in children. 2. Severe malaria]. *Med Trop (Mars)* 62(6): 657-64.

[19] Marsh K, Marsh VM, Brown J, Whittle HC, Greenwood BM (1988). Plasmodium falciparum: the behavior of clinical isolates in an in vitro model of infected red blood cell sequestration.*Exp Parasitol* 65(2):202-8.

[20] Taylor HM, Triglia T, Thompson J, Sajid M, Fowler R, Wickham ME, Cowman AF, Holder AA. (2001). Plasmodium falciparum homologue of the genes for Plasmodium vivax and Plasmodium yoelii adhesrive proteins, which is transcribed but not translated. *Infect Immun*. Jun; 69 (6): 3635-45.

[21] Mazier D, Nitcheu J and Idrissa-Boubou M. (2000). Cerebral malaria and immunogenetics. *Parasite Immunol* 22(12): 613-23.

[22] Brown HC, Chau TT, Mai NT, Day NP, Sinh DX, White NJ, Hien TT, Farrar J, Turner GD. (2000). Blood-brain barrier function in cerebral malaria and CNS infections in Vietnam. *Neurology*. Jul 12;55(1):104-11.

[23] Hunt NH, Grau GE. (2003). Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. *Trends Immunol*; 24:491-499.

[24] Pino P, Vouldoukis I, Kolb JP, Mahmoudi N, Desportes-Livage I, Bricaire F, Danis M, Dugas B, Mazier D. (2003). Plasmodium falciparum--infected erythrocyte adhesion induces caspase activation and apoptosis in human endothelial cells. *J Infect Dis*. Apr 15;187 (8):1283-90. Epub 2003 Apr 2.

[25] Touré FS, Ouwe-Missi-Oukem-Boyer O, Bisvigou U, Moussa O, Rogier C, Pino P, Mazier D, Bisser S. (2008). Apoptosis: a potential triggering mechanism of neurological manifestation in Plasmodium falciparum malaria. *Parasite Immunol*. Jan;30 (1):47-51.

[26] Combes V, Coltel N, Faille D, Wassmer SC, Grau GE. (2006). Cerebral malaria: role of microparticles and platelets in alterations of the blood-brain barrier. *Int J Parasitol*. May 1;36(5):541-6.

[27] Bisser S, Ouwe-Missi-Oukem-Boyer ON, Toure FS, Taoufiq Z, Bouteille B, Buguet A, Mazier D. (2006). Harbouring in the brain: A focus on immune evasion mechanisms and their deleterious effects in malaria and human African trypanosomiasis. *Int J Parasitol*. May 1;36(5):529-40.

[28] Siau A, Toure FS, Ouwe-Missi-Oukem-Boyer O, Ciceron L, Mahmoudi N, Vaquero C, Froissard P, Bisvigou U, Bisser S, Coppee JY, Bischoff E, David PH, Mazier D. (2007). Whole-transcriptome analysis of Plasmodium falciparum field isolates: identification of new pathogenicity factors. *J Infect Dis*. Dec 1;196(11):1603-12.

[29] Schofield FD, (1955). Two cases of loiasis with peripheral nerve involvement. *Trans. Roy. Soc. Trop. Med. Hyg*. 49 : 588-589.

[30] Hulin C, et al., (1994). Atteinte pulmonaire d'évolution favorable au cours d'une filariose de type *Loa loa*. *Bull. Soc. Path. Ex*. 87: 248-250.

[31] Okell LC, Ghani AC, Lyons E, Drakeley CJ. (2009). Submicroscopic infection in Plasmodium falciparum-endemic populations: a systematic review and meta-analysis. *J Infect Dis*. Nov 15;200 (10):1509-17.

[32] Snounou, G. S., S. Viriyakosol, X. P. Zhu, W. Jarra, L. Pinheiro, V. E. Do Rosario, S. Thaithong, and K. N. Brown (1993). High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol. Biochem. Parasitol*. 61:315–320.

[33] Mixson-Hayden T, Lucchi NW, Udhayakumar V. (2010). Evaluation of three PCR-based diagnostic assays for detecting mixed Plasmodium infection. *BMC Res Notes*. Mar 31;3:88.

[34] Jarra W, Snounou G. (1998). Only viable parasites are detected by PCR following clearance of rodent malarial infections by drug treatment or immune responses. *Infect Immun*. Aug;66(8):3783-7.

[35] Cheng Q, Lawrence G, Reed C, Stowers A, Ranford-Cartwright L, Creasey A, Carter R, Saul A. Measurement of Plasmodium falciparum growth rates in vivo: a test of malaria vaccines. *Am J Trop Med Hyg*. 1997 Oct;57(4):495-500.

[36] Filisetti D, Bombard S, N'Guiri C, Dahan R, Molet B, Abou-Bacar A, Hansmann Y, Christmann D, Candolfi E. Prospective assessment of a new polymerase chain reaction target (STEVOR) for imported Plasmodium falciparum malaria. *Eur J Clin Microbiol Infect Dis*. 2002 Sep;21(9):679-81. Epub 2002 Sep 6.

[37] Oyedeji SI, Awobode HO, Monday GC, Kendjo E, Kremsner PG, Kun JF. (2007) Comparison of PCR-based detection of Plasmodium falciparum infections based on single and multicopy genes. *Malar J.* Aug 16;6:112.

[38] Farcas GA, Zhong KJ, Mazzulli T, Kain KC. (2004). Evaluation of the RealArt Malaria LC real-time PCR assay for malaria diagnosis. *J Clin Microbiol*. Feb;42(2):636-8.

[39] Lee MA, Tan CH, Aw LT, Tang CS, Singh M, (2002) Lee SH, Chia HP, Yap EP. Real-time fluorescence-based PCR for detection of malaria parasites. *J Clin Microbiol*. Nov;40(11):4343-5.

[40] Touré FS, et al., (1997a). Species-specific sequence in the repeat 3 region of the gene encoding a putative *Loa loa* allergen: a diagnostic tool for occult loiasis. *Am J Trop Med Hyg*. 56: 57-60.

[41] Touré FS, Odile Bain, Eric Nerrienet, et al. (1997b). Detection of *Loa loa*-specific DNA in blood from occult infected individuals. *Exp Parasitol*. 86: 163-70.

[42] Ouwe-Missi-Oukem-Boyer ON, Hamidou AA, Sidikou F, Garba A, Louboutin-Croc JP. (2005). The use of dried blood spots for HIV-antibody testing in Sahel]. *Bull Soc Pathol Exot*. Dec;98(5):343-6. French.

[43] Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE (1995). Pyrimethamine and proguanil resistance-conferring mutations in Plasmodium falciparum dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *Am J Trop Med Hyg* 52: 565–568.

[44] Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN. (1993). High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol*. Oct;61(2):315-20.

[45] Mockenhaupt FP, Ulmen U, von Gaertner C, Bedu-Addo G, Bienzle U.(2002). Diagnosis of placental malaria. *J Clin Microbiol*. Jan;40(1):306-8.

[46] Touré FS, Mezui-Me-Ndong J, Ouwe-Missi-Oukem-Boyer O, Ollomo B, Mazier D, Bisser S. (2006). Submicroscopic Plasmodium falciparum infections before and after sulfadoxine-pyrimethamine and artesunate association treatment in Dienga, Southeastern Gabon. *Clin Med Res*. Sep;4(3):175-9.

[47] Rogier C, Commenges D, Trape JF (1996). Evidence for an age-dependent pyrogenic threshold of Plasmodium falciparum parasitemia in highly endemic populations. *Am J Trop Med Hyg*. Jun;54(6):613-9.

[48] Giha HA, A-Elbasit IE, A-Elgadir TM, Adam I, Berzins K, Elghazali G, Elbashir MI. (2005). Cerebral malaria is frequently associated with latent parasitemia among the semi-immune population of eastern Sudan. *Microbes Infect*. Aug-Sep;7(11-12):1196-203.

[49] Bouyou-Akotet MK, Nzenze-Afene S, Ngoungou EB, Kendjo E, Owono-Medang M, Lekana-Douki JB, Obono-Obiang G, Mounanga M, Kombila M. (2010). Burden of malaria during pregnancy at the time of IPTp/SP implementation in Gabon. *Am J Trop Med Hyg*. Feb;82(2):202-9.

[50] Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR. (2002). Epidemiology of drug-resistant malaria. *Lancet Infect Dis*. Apr; 2 (4):209-18.

[51] Duraisingh MT, Drakeley CJ, Muller O, Bailey R, Snounou G, Targett GA, Greenwood BM, Warhurst DC (1997). Evidence for selection for the tyrosine-86 allele of the pfmdr 1 gene of Plasmodium falciparum by chloroquine and amodiaquine. *Parasitology*. Mar;114 (Pt 3):205-11.

[52] Coleman RE, Kumpitak C, Ponlawat A, Maneechai N, Phunkitchar V, Rachapaew N, Zollner G, Sattabongkot J. (2004). Infectivity of asymptomatic Plasmodium-infected human populations to Anopheles dirus mosquitoes in western Thailand. *J Med Entomol*. Mar;41(2):201-8.

[53] Nwakanma D, Kheir A, Sowa M, Dunyo S, Jawara M, Pinder M, Milligan P, Walliker D, Babiker HA (2008). High gametocyte complexity and mosquito infectivity of Plasmodium falciparum in the Gambia. *Int J Parasitol*. Feb;38(2):219-27. Epub 2007 Jul 18.

[54] Karl S, Gurarie D, Zimmerman PA, King CH, St Pierre TG, Davis TM. (2011). A sub-microscopic gametocyte reservoir can sustain malaria transmission *PLoS One*; 6 (6):e20805. Epub 2011 Jun 14.

[55] Liljander A, Chandramohan D, Kweku M, Olsson D, Montgomery SM, Greenwood B, Färnert A (2010). Influences of intermittent preventive treatment and persistent multiclonal Plasmodium falciparum infections on clinical malaria risk. *PLoS One*. Oct 27;5(10):e13649.

[56] Touré FS, Mavoungou E, Kassambara L, Williams T, Wahl G, Millet P, Egwang TG (1998). Human occult loiasis: field evaluation of a nested polymerase chain reaction assay for the detection of occult infection. *Trop Med Int Health*. 3: 505-511.

[57] Touré FS, Deloron P, Egwang TG, Wahl G (1999a). [Relationship between the intensity of *Loa loa* filariasis transmission and prevalence of infections] *Med Trop*. 59: 249-52. French.

[58] Touré FS, Ungeheuer MN, Egwang TG, Deloron P. (1999b). Use of polymerase chain reaction for accurate follow-up of *Loa loa* experimental infection in *Mandrillus sphinx*. *Am J Trop Med Hyg*. 61: 956-9.

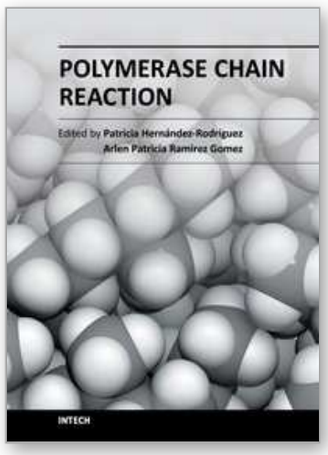
[59] Pinder M, Everaere S, Roelants GE (1994). Loa loa: immunological responses during experimental infections in mandrills (Mandrillus sphinx). *Exp Parasitol*. Sep; 79(2):126-36.

[60] Pinder M Dupont A, Egwang TG. (1988). Identification of a surface antigen on *Loa loa* microfilariae the recognition of which correlates with the amicrofilaremic state in man. *Jour. Immunol*. 141 : 2480-2486.

[61] Akué JP, Hommel M., Devaney E. (1997). High levels of parasite- specific IgG1 correlate with the amicrofilaremic state in *Loa loa* infection. *Jour. Infect. Dis*. 175 : 158-163.

[62] Baize S, Wahl G, Soboslay PT, Egwang TG, Georges AJ. (1997). T helper responsiveness in human *Loa loa* infection: defective specific proliferation and cytokine production by CD4+ T cells from microfilaremic subjects compared with amicrofilaremics. *Clin. Exp. Immunol*. 108 : 272-278.

[63] Leroy E, Baize S, Wahl G, Egwang TG, Georges AJ. (1997). Experimental infection of a nonhuman primate with Loa loa induces transient strong immune activation followed by peripheral unresponsiveness of helper T cells. *Infection Immunity*. 65 (5) : 1876-1882.



Polymerase Chain Reaction

Edited by Dr Patricia Hernandez-Rodriguez

ISBN 978-953-51-0612-8

Hard cover, 566 pages

Publisher InTech

Published online 30, May, 2012

Published in print edition May, 2012

This book is intended to present current concepts in molecular biology with the emphasis on the application to animal, plant and human pathology, in various aspects such as etiology, diagnosis, prognosis, treatment and prevention of diseases as well as the use of these methodologies in understanding the pathophysiology of various diseases that affect living beings.

InTech Europe

University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen