Use of a Locked-Nucleic-Acid Oligomer in the Clamped-Probe Assay for Detection of a Minority Pfcr K76T Mutant Population of Plasmodium falciparum

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Given the emergence of drug resistance and the high rate of polyclonal microorganism infections, the availability of a fast and sensitive test to detect minority populations of resistant microorganisms would be an improvement in the diagnosis of infectious diseases. A clamped-probe real-time PCR assay to diagnose the Plasmodium falciparum K76T mutation in clone populations was developed, using a wild-type-specific locked-nucleic-acid-containing oligomer to suppress wild-type PCR amplification and to enhance melting analysis with a mutation-specific detection probe.

In the last few decades, the emergence of drug resistance has become a major problem for the treatment of various infectious diseases. Bacterial, viral, or parasitic infections are often polyclonal (4, 10, 12, 25, 27). Drug-resistant individuals may hide among a sensitive population and the identification of a resistant clone may have implications for epidemiology and treatment. Molecular diagnosis of resistant microorganisms by mutation detection is a routine examination in virology (17, 20) and has started to be used in bacteriology (5, 19, 23) and parasitology (24). Recent virology studies have indicated that the presence of minority drug-resistant variants may be an independent predictor of treatment failure (8, 9). The emergence of nosocomial infections caused by drug-resistant bacteria also represents a significant threat for debilitated patients (22). As a consequence, a need exists to have a fast and sensitive diagnostic test to identify minority populations of resistant organisms. In order to improve the efficacy of such a genotype-assisted diagnosis, methods have been developed to lower the detection threshold of minority populations among a wild-type one. This trend was demonstrated by two recent studies. First, a rapid and accurate method of duplex real-time PCR used to detect ampicillin-resistant Enterococcus faecium, a major cause of nosocomial infections, was recently published (19). Second, an RNA heteroduplex generator tracking assay elicited a decrease in the detection threshold of type 1 human immunodeficiency virus mutant populations from 20 to 0.5% (12). Concerning the diagnosis of Plasmodium falciparum malaria, the detection of a minority population has become important with the discovery of Pfcrt K76T mutation, which has been correlated with in vitro chloroquine resistance (1, 7, 26).

Chloroquine has proved to be one of the most successful drugs ever deployed against P. falciparum malaria, mainly because it is well tolerated and available at a very low cost. Over the last three decades, P. falciparum chloroquine resistance has significantly increased, and many countries have discarded this drug from the antimalarial weaponry. In these areas, it has recently been observed that the reappearance of chloroquine-sensitive P. falciparum strains has followed chloroquine withdrawal (15, 21). This phenomenon, if confirmed in the future, could make reusing chloroquine as the first line treatment of malaria possible (18, 21), e.g., in combination with artemisinin derivatives. However, such a therapeutic option would imply public health surveillance based upon sensitive techniques. The published real-time PCR mutation detection method (24) was combined with locked-nucleic-acid (LNA) residues containing clamping oligomer specific for the wild-type sequence of the P. falciparum Pfcrt gene to enhance the detection of a minority mutant population.

MATERIALS AND METHODS

Reference P. falciparum strains. A chloroquine-resistant strain, FcM29-Cameroon, mutated codon 76 of Pfcrt, and an F32-Tanzania chloroquine-sensitive isolate, wild-type for codon 76 of Pfcrt, were maintained in vitro by continuous culture with human O red blood cells (Regional Blood Bank, Toulouse, France) diluted to 1% hematocrit in an RPMI 1640 medium (Gibco and Invitrogen, Cergy Pontoise) containing 5% of human serum. The cultures were synchronized to ring stage by lysis with 5% D-Sorbitol (Merck, Darmstadt, Germany) in order to have one nucleus per parasite.

Reference P. falciparum-infected patients. A total of 106 patients with a positive diagnostic of P. falciparum by an optical method (quantitative bulky coat and thin-smear examination) and confirmed by PCR (6) were tested. For each patient, 5 ml of whole blood was collected in an EDTA vial, and all laboratory testing was done on the same sample. The present study used the routine sample, so patients’ informed consent was not required, according to a general policy of the Board for Clinical Research of the University Hospital.

Clamping probe assay for detection of the Pfcrt K76T mutation. DNA was extracted by using a High-Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol. Primers and detection probe were as published previously (24), using an internal LC640-labeled primer and an adjacent fluorescence-labeled mutation-specific sensor probe on the complementary strand, spanning codons 76 to 83. The competing wild-type specific LNA oligomer covered codons 75 to 81; the 3’ terminus was

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phosphorylated to prevent extension by Taq polymerase. Two different clamping oligomers were tested, containing either 6 (ClampLNAdt1) or 10 (ClampLNAdt2, all dT bases substituted) dT-LNA residues (Plas iLCS) and of the antisense primer (Plas R), 0.4 μM fluorescent-labeled Sensor [C], 3.5 mM MgCl₂, 2 μM ClampLNAdt2, the LightCycler DNA Master Hybridization reagent (Roche Diagnostics), and 5 μl of DNA in a final volume of 20 μl. After an initial denaturation step (8 min at 95°C), 50 amplification cycles were performed (15 s at 95°C, 25 s at 50°C, and 7 s at 76°C). Melting analysis was performed by holding the denaturation reaction at 95°C for 1 min and hybridization at 40°C for 2 min, followed by monitoring the fluorescence at 640 nm from 40 to 65°C, with a temperature transition rate of 0.1°C per s.

**Results and Discussion**

**LNA-based clamped-probe assay.** Peptide-nucleic acid (PNA) oligomers were used for the clamped-probe assay detection of K-ras (3) and ABL (14) mutations. We used LNA-containing clamping oligomers with six or ten dT-LNA residues. LNA is a synthetic RNA analogue with a methylene bridge between the ribose 2′-oxygen and the 4′-carbon atoms, “locking” the sugar ring in a fixed 3′-endo conformation, which is the preferable structure of RNA-DNA hybrids (2, 13). LNA bases are indicated in bold type.

Concerning the mutant sequence, there was only a small difference (2.5% of mutant sequence and 97.5% of wild-type sequence).

The ability of the method to detect a minority mutant population in a wild-type one was assessed by testing increasing dilutions of the mutant FcM2 strain on codon 76 contains 18 of 21 weak AT bases (85%). The introduction of LNA bases is a powerful tool to get higher-melting probes of moderate length. Due to the symmetry of the sequence (AA AATTTT), we did not substitute any dA bases for their LNA analogues to prevent dimerization of the clamping probe. Furthermore, the substitution of only one type of base saved money since the LNA monomers were relatively expensive. Of the two different clamping probes tested, containing six dT-LNA residues (ClampLNAdt2) (Tm = 60°C [calculation according to the method of Exiqon]) and the other 10 dT-LNA residues (ClampLNAdt1) (Tm = 75°C), the second showed a better discrimination power (data not shown).

A four-step PCR program as used for the PNA-based clamped-probe assay (3, 14) was not necessary and a conventional three-step PCR worked. The best differentiation was obtained with an extension temperature of 76°C, a level at which the wild-type allele still bound, whereas the polymerase readthrough for the mutated target sequence was impeded (data not shown).

**Dose effect of the LNA oligomer (ClampLNAdt2).** LNA oligomer concentration was tested in the range of 1 to 4 μM (final concentration). Figure 2 shows a dose effect for a 1:40 dilution (2.5% of mutant sequence and 97.5% of wild-type sequence).

Concerning the mutant sequence, there was only a small difference on the melting curve between the three concentrations:

**TABLE 1. Description of oligonucleotides**

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<tr>
<th>Oligonucleotide</th>
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<th>Tm (°C)</th>
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<td>242–222</td>
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* Corresponding positions in the wild-type sequence of the P. falciparum Pfcrt gene (previously submitted to GenBank under accession no. AF495378).
FIG. 2. Dose effect of LNA clamp (Clamp$_{LNA}^{LNA_{dR}}$) concentration on PCR product amplification. Different LNA clamp concentrations were tested at a 1:40 ratio mixture of the K76T MS to WTS (MS [2.5%] and WTS [97.5%] for 1% parasitemia). (A to E) Optical reading of the PCR products melting curve. The left peak corresponds to a K76T WTS population and the right peak corresponds to a K76T MS population. (A) Without LNA clamp; (B) 1 μM; (C) 2 μM; (D) 3 μM; (E) 4 μM. The LNA clamp concentration worked with a dose effect on MS detection and WTS inhibition. (F) Real-time PCR product quantification curve. The PCR product amplification decreased when the LNA clamp concentration increased.
2, 3, and 4 μM. In addition, the same concentrations tested on a 100% mutant sample showed that the LNA oligomer also decreased amplification of this allele. This inhibition caused by the high affinity between LNA bases and DNA was proportional to the LNA oligomer concentration (data not shown). The optimal concentration of the LNA oligomer had to be adjusted between a strong inhibition of the wild-type background and a weak inhibition of mutant amplification. Based on these results, we used a 2 μM LNA oligomer concentration that showed a good signal for the mutated allele.

**Sensitivity of the clamping probe assay.** The present method, using the LNA oligomer (ClampLNAΔt2), was tested for different mixture ratios of a mutant population among a wild-type one ranging from 1:2 to 1:1,600 when the parasitemia was constantly 1% or 41,000 parasites/μl (Fig. 3). The LNA clamping probe assay detected a mutated population diluted to 1:1,600 in the wild type, i.e., 0.063% (26 parasites/μl) of mutated plasmodia in 99,937% (40,974 parasites/μl) of wild-type parasites. The sensitivity of the present assay was identical when a mixture displaying a 0.1% (4,100 parasites/μl) parasitemia was tested (data not shown). Dilutions greater than 1:1,600 were tested, but the optical reading of the curves was awkward, so the workers barely distinguished positive results from the negative reference sample.

Finally, the present method increased by 32-fold the power of detection of a minority mutant population compared to the conventional real-time PCR method (24) that had showed a 2% sensitivity (1:50 dilution with a 1% parasitemia).

**Reproducibility, repeatability, and robustness of the system.** Reproducibility was assessed by using three different samples of each mixture and ten samples from infected patients: five WTS and five mixed strains (WTS+MS) and by testing each
sample three times in different PCR runs. The results were similar for each sample with the same mixture ratio, even for the lower concentrations of mutant allele and for all the patient samples (data not shown). Repeatability was assessed with the same samples as those used for reproducibility but which were tested three times in the same PCR run. The results were similar for each sample (data not shown). Robustness was also checked by testing 106 samples from infected patients that were found to be either WTS, MS, or mixed (data not shown).

The chemosensitivity assays of Plasmodium isolates, performed either by classical in vitro techniques or by standard molecular detection of a given mutation involved in the resistance status, concerned mainly the majority clone. Malaria patients are infected by polyclonal Plasmodium populations (4, 10), of which one or more may be mutated. The LNA-based clamped-probe assay for the diagnosis of minority resistant populations will improve the precocity of detecting such minority populations; thus, improving control of emerging resistant clones by fast adaptation of therapeutic policy. In addition, this new technique could improve our knowledge of polyclonal Plasmodium population dynamics in humans and in anophelines. Due to the flexibility of the individual substitution for LNA bases, designing new clamped-probe assays will be much easier. In fields other than malaria, this type of test might improve the diagnosis of bacterial, viral, or parasitic infections for the monitoring of minimal residual diseases and also for the analysis of complex mixtures such as food and environmental samples. Apart from the clamped-probe assay, LNA bases could be inserted in primers to allow modification of their Tm or their lengths to improve PCR in some DNA areas where design conditions are difficult.

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