ONE YEAR'S EXPERIENCE WITH THE POLYMERASE CHAIN REACTION AS A ROUTINE METHOD FOR THE DIAGNOSIS OF IMPORTED MALARIA

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Abstract. Given the problems encountered in westernized countries with the laboratory diagnosis of malaria, namely sensitivity of the conventional methods and detection of mixed infections, a polymerase chain reaction (PCR)–based diagnostic method has been developed and routinely used. The PCR used two sets of primers to simultaneously detect any infection due to the genus Plasmodium, or to the species P. falciparum. The PCR results were available within six hours. Five hundred twenty-nine patients were tested, of whom 136 were found positive by the PCR, and only 104 by the quantitative buffy coat (QBC™) method. The 32 discrepancies were analyzed on the basis of the clinical data, and technical, molecular, and sequencing findings to ascertain the presence of Plasmodium DNA. The PCR-based diagnosis of malaria appeared to be a useful tool that was suitable as a second-line method when the results of conventional techniques were negative in patients presenting a syndrome consistent with malaria, as well as yielding an accurate species identification.

INTRODUCTION

Since the mid 1980s, imported malaria in westernized countries has become a growing and worrisome problem due to the spread of drug resistance and to the increase in both travel to tropical countries and arrival of immigrants from malarious areas. In 1998, a report from the National Center for Imported Diseases in France indicated 5,940 ± 88 cases of imported malaria were distributed as follows: 82.3% of the infections were caused by Plasmodium falciparum, 6.9% by P. vivax, 5.8% by P. ovale, and 1.2% by P. malariae; 2.2% of the infections were mixed and 1.1% were due to P. spp.1

In such patients, a diagnosis can be impaired by the presence of unusual clinical features and/or of a low parasitemia,2 which are often caused by a prophylaxis not properly followed, or a partially efficient drug.3,4 In the largest French hospitals and in those run by the French Army, the emergency laboratory diagnosis of malaria has greatly improved during the last decade due to the combined use of two types of tests. The first test, which is based on the optical detection of parasites, is the quantitative buffy coat (QBC™) system.5 This system, which is also called “rocket diagnosis,” was introduced in the early 1990s. It was later coupled with immunologic methods such as the ICT™ or Parasight™6,7 dipstick assays, which detect P. falciparum histidine-rich protein-2. However, these assays give only in a diagnosis of malaria and infection with P. falciparum.

Results obtained in an emergency laboratory should be carefully checked and verified by microscopy examination of thin and/or thick blood smears. This conventional method, albeit considered the “gold standard,” is time-consuming, requires well-trained technicians, and has poor sensitivity when only thin smears are examined.8 Moreover, species diagnosis, especially by examination of thick blood smears, is sometimes difficult, thus leading to unreliable results. This was encountered with specimens from patients who were infected with P. falciparum strains that had intermediate sensitivity to a chemoprophylactic agent, e.g., the widely used combination of chloroquine plus proguanil, or from patients who treated themselves with an insufficient dosage of an otherwise efficient drug, e.g., halofantrine.9 Moreover, for non-falciparum species, parasitemia can be very low (only one parasite on several slides), and the appearance of this single parasite can be equivocal. Therefore, an obvious need exists for a routine diagnostic method that displays both high sensitivity and specificity, and whose results would not depend on the experience of the individuals performing this method.

Polymerase chain reaction (PCR)–based methods have been a recent development in the molecular diagnosis of malaria. Since 1990, several experimental assays have been reported that use various primers, and extraction and detection techniques.9–12 Several reports have shown that the PCR had a higher sensitivity than examination of thin blood smears, especially in cases with low parasitemia or mixed infections.13–15 The PCR was also found to be more sensitive than the QBC™ method and dipstick assays (sensitivity = 100% versus 88%).16 Compared with examination of thin blood smears, the specificity of the PCR was excellent,12,17–20 and greater than that of the QBC™ or dipstick assays.15

While the PCR appears to have overcome the two major problems of the diagnosis of imported malaria, namely sensitivity and specificity, the routine feasibility of this method still remained to be assessed. Most of the previously reported studies were performed on selected populations that were batch-tested against a reference technique in a single round.6,17–19 No studies have tested daily use of the PCR for routine diagnosis of malaria for one year in more than 500 patients.

The aim of the present study was develop and routinely use for 12 months a PCR-based method for the diagnosis of imported malaria. The results obtained with this molecular technique were evaluated against those from both conventional microscopic examination and the QBC™ method. Since the QBC™ method is routinely used in the laboratory and has been reported as being more sensitive than the thick blood smear,12,15,18–20 it was chosen as the reference microscopy method. The daily routine use of the PCR was also assessed for reproducibility, compliance with good laboratory practices,21 and operational costs. The use (first- or second-line) of the PCR in comparison with other diagnostic methods was also assessed.

MATERIALS AND METHODS

Patients. All patients attending the University Hospitals of Toulouse from October 1999 to September 2000 for whom a malaria diagnosis was required were included in the study. Five hundred twenty-nine patients were tested (59.2% males
and 40.8% females). Their ages ranged from 2 days to 90 years (mean = 35.7 years). Five milliliters of whole blood was collected from each patient into a vial containing EDTA, and all laboratory tests were performed on the same sample. Since the study used a routine sample, patients’ informed consent was not required, according to the general position of the Board for Clinical Research of the University Hospital.

Conventional microscopy. Thin smears were made from the blood samples and were stained with May-Grünwald-Giemsa. The stained slides were read for at least 20 minutes by the senior clinical biologists of the malaria unit of the Department of Parasitology. Since the malaria diagnosis was performed daily, a pool of four biologists was used to read the slides.

QBC™ method. The QBC™ test (Becton-Dickinson, Le Pont de Claix, France) was performed on blood samples according to manufacturer’s instructions. Test results were read for at least 10 minutes by the members of the above-mentioned pool of senior clinical biologists.

Immunodiagnosis. A serodiagnosis of malaria was performed for some cases by immunofluorescence. The assay used *P. falciparum*-parasitized human erythrocytes (Falciparum-spott™ Bioméreux, Marcy l’Etoile, France) according to manufacturer’s instructions. The cut-off value was 1:100.

PCR. Laboratory organization. The PCR unit worked on the basis of five days of labor per week. As a result, approximately 250 PCR rounds (a total of 623 tests) were performed for 529 patients. An average of 2.48 PCR-based diagnoses were performed daily.

To avoid any contamination, the PCR assay was carried out in three separate rooms: one for preparation of the reaction mixture, one for the extraction of DNA, and one for the amplification and detection of the products. One technician was assigned to extract the DNA and do the amplification, and another conducted the electrophoresis of the amplified products. A pool of four clinical laboratory technicians was in charge of the routine molecular diagnosis. The sequencing was done by two of the authors (R.F and A.B.)

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Molecular diagnosis of malaria was conducted as follows. Systematically, at the first step, two PCR assays were simultaneously made, one that detected parasites of the genus *Plasmodium* (PspC PCR), and one that specifically detected *P. falciparum* (Pf PCR). Both tests were coupled with an internal amplification control of the human β-globin gene to check for the absence of inhibitors. For every sample displaying only a positive PspC PCR, a second set of three PCRs was subsequently performed one day later. These additional PCR assays were specific for *P. malariae* (Pm PCR), for *P. ovale* (Po PCR), and for *P. vivax* (Pv PCR), respectively.

Patients simultaneously displaying a negative PspC PCR and Pf PCR result, along with a positive test result for the β-globin PCR, were classified as malaria free. Those exhibiting a positive result in the PspC PCR and/or the Pf PCR were classified as malaria infected. The Pm PCR, Po PCR, and Pv PCR were then performed to detect non-falciparum or mixed infections. In case of a discrepancy between the PspC PCR and Pf PCR, or of a negative result in the β-globin control assay, extraction of DNA and the PCRs were repeated.

PCR primers. The choice of the set of primers was determined by the diagnosis strategy. The strategy was to first detect any malarial infection using genus-specific oligonucleotides, then detect any falciparum infection (the only common malarial emergency), and then rule out the presence of PCRs inhibitors by detecting the human β-globin gene. Both specificity and sensitivity of the selected primers had previously been ascertained by pilot studies.

The following primers were used: for the PspC PCR, primers L1 (5’-GAC CTG CAT GAA AGA TG-3’), L2 (5’-GTA TCG CTT TAA TAG GCG-3’) based on the Cox I mitochondrial gene;20 for the Pf PCR, primers Pf1 (5’-GGA ATG TTA TTG CTA ACA C-3’) and Pf2 (5’-AAT GAA GAG CTG TGT ATC-3’);21 for the human β-globin gene, primers BG07 (5’-GGT TGG CCA ATC TAC TCC CAG G-3’) and BG08 (5’-TGG TCT CCT TAA ACC TCT GT-3’);22 for the Pm PCR, primers PLF (5’-AGT GTG TAT CAA TCG AGT TT-3’) and MAR (5’-GCC CTC CAA TTG CTT TCT-3’) based on the SSUrRNA gene;17 for the Po PCR, primers PLF and OVR (5’-GCA TAA GGA ATG CAA AGA ACA AGA G-3’);17 and for the Pv PCR, primers Pv1 (5’-CAC CAT TAA GTA CAT CAC-3’) and Pv2 (5’-TGG TAA CAC AAC TCC AAT-3’) based on the Cox I mitochondrial gene.20

PCR amplification. Amplification was carried out in a DNA thermal cycler type 480™ (Perkin-Elmer, Courtaboeuf, France). Five microliters of extracted DNA were resuspended in 95 µl of a PCR buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 200 µM of each of the deoxynucleoside triphosphates, 0.5 units of Taq Gold™ (Roche Diagnostics), and 0.4 µM of primers L1/L2, BG07/BG08 (PspC PCR) and Pf1/Pf2 (Pf PCR) or 0.2 µM of primers PLF/MAR (Pm PCR), PLF/OVR (Po PCR), or Pv1/Pv2 (Pv PCR).

The amplification step included a seven-minute denaturation cycle at 95°C and 40 one-minute cycles at 90°C; a two-minute annealing step at 56°C (PspC PCR and Pf PCR), 60°C (Pm PCR), 51°C (Po PCR), or at 53°C (Pv PCR), and a one-minute elongation step at 72°C. In the final cycle, an additional five-minute elongation step at 72°C was included. The thermal cycler temperature remained at 15°C after the final cycle, until the samples were removed.

Detection of PCR products. Amplified PCR products were detected by electrophoresis. A standard 1.5% agarose gel was loaded with 10-µl samples of the PCR mixtures. Samples were subjected to electrophoresis and stained using a solution of ethidium bromide (0.5 µg/ml). Each gel was visualized with ultraviolet light and photographed.

The PCR products varied according to the type of assay. A 595-basepair fragment was detected for the genus *Plasmodium*. A 422-basepair fragment was detected for *P. falciparum*, an 871-basepair fragment for *P. vivax*, a 463-basepair fragment for *P. ovale*, and a 269-basepair fragment for *P. malariae*.504
malaria (Figure 1). Every PCR included one negative control sample (DNA replaced with water), one positive control for P. falciparum, and one positive control for Pspp (mostly P. vivax).

Sequencing of DNA. Direct sequencing of PCR products was carried out to check for their quality, or when a discrepancy between PCR and optical microscopy occurred in species diagnosis. Sequencing reactions were performed using an ABI Prism Sequencer™ (Perkin-Elmer). The DNA sequences were compared with data in GenBank.

Accession numbers to GenBank. The GenBank references of the nucleotide sequences of the genes used for the diagnosis were M76611 (P. falciparum), 5S4897 (P. malariae), L48986 (P. ovale), and X13926 (P. vivax).

RESULTS

Contamination was observed during the study in February 2000. This problem was resolved by moving the amplification/detection procedures to a new laboratory more distant from the other two laboratories, albeit in the same building, and by reinforcing discipline among the team of technicians (no one was permitted to enter another room used for the PCR).

Results from both the Pspp PCR and Pf PCR were available every working day within six hours. The cost of a diagnosis (Pspp PCR and Pf PCR), including reagents, technicians’ labor, and depreciation of the equipment, was estimated to be approximately 13 euros when more than one assay was performed (19 euros for a single test).

Thirty-two (6.23%) malaria infections detected by the PCR were not detected by the QBTC™ test. Therefore, the PCR was compared with the QBTC™ test for only 529 patients. The results are shown in Table 1. All 32 patients found to be malaria negative by the QBTC™ test were also negative by conventional microscopy. These results indicate that the sensitivity of the PCR is maximized by two sets of PCR, the only way to determine whether this assay, when routinely used for one year, could maintain interserial sensitivity and specificity greater than those of conventional methods, coupled with a delivery of the result within a few hours.

The design of laboratory procedures, as well as the selection of primers, was of crucial importance in the PCR procedure. The combined use of two sets of previously tested primers, which simultaneously detected the genus Plasmodium and the species P. falciparum, resulted in the diagnosis of any malarial infection in one day. Coupling of two independent PCRs at the same temperature of hybridization increased the safety level of the diagnosis due to the use of two independent but related reactions. Moreover, the amplification of the β-globin gene in the procedure ensured the absence of any inhibitor. During this 12-month study, no inhibitor was detected.

In 529 patients tested over a 12-month period, the PCR constantly exhibited a greater sensitivity than the QBTC™ method, which is the most sensitive optical method (Table 1). It could therefore be inferred that the interserial sensitivity of the PCR was maintained during the one-year study period. No discrepancy between negative PCR results and positive optical method results was observed. With regard to the 32 positive Pspp or Pf PCR samples that were negative by the QBTC™ method (Table 1), a diagnosis of malarial infections could not be ascertained by another method other than PCR. Nevertheless, some findings indicated the likely presence of Plasmodium DNA.

All patients tested were suspected of having malaria based on clinical and epidemiologic data. DNA hybridization is believed to be a more efficient method in recognizing a specific DNA sequence. In this study, two hybridizations were simultaneously performed, and each positive case was found by four different hybridizations steps in two different PCRs. If the specificity is maximized by two sets of PCR, the only way

![Figure 1. Polymerase chain reaction showing positive controls (a) and positive patients (b). Pspp = genus Plasmodium; Pf = P. falciparum; Pm = P. malariae; Po = P. ovale; Pv = P. vivax.](Image)
to have a false-positive diagnosis would be if contamination occurred. However, when a PCR diagnosis is performed according to good laboratory practices, as in the present study, carryover contamination is easily excluded by the use of negative controls. The PCR product was sequenced when a discrepancy in parasite identification between optical diagnosis and the PCR method was found. Sequencing data, when compared with those from GenBank, were in perfect accordance. It therefore appears likely that these 32 cases, which were positive only by the molecular method, were true malaria infections, regardless of the clinical findings.

For the 32 discrepancies, further PCR tests resulted in the following distribution of infections: 26 *P. falciparum*, two *P. malariae*, two *P. ovale*, and two *P. vivax* infections. A retrospective analysis of the medical records of these patients was carried out, and these 32 cases were divided into four groups.

The 14 patients in group I (12 *P. falciparum*, 1 *P. ovale*, and 1 *P. vivax*) were previously treated for malaria. Due to the type of recruitment (patients attending an emergency care unit at night), miscommunication occurred between the clinician and the Laboratory of Parasitology. Information regarding previous treatment was not available by the time of the sample, or the use of a legal medical identity by an illegal immigrant and the Laboratory of Parasitology. Information regarding previous treatment was not available by the time of the sample, or the use of a legal medical identity by an illegal immigrant, should therefore be considered.

The five patients in group III (3 *P. falciparum*, 1 *P. malariae*, and 1 *P. ovale*) were travelers returning from countries endemic for malaria. They attended the emergency care unit of the University Hospital due to a clinical syndrome consistent with malaria. Profiles of these patients are shown in

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### Table 2

Secondary species identification by the polymerase chain reaction (PCR) in 102 cases diagnosed by examination of thin blood smears

<table>
<thead>
<tr>
<th>Thin smear result</th>
<th>Single <em>P. falciparum</em> infection</th>
<th>Single <em>P. malariae</em> infection</th>
<th>Single <em>P. ovale</em> infection</th>
<th>Single <em>P. vivax</em> infection</th>
<th>Mixed infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single <em>P. falciparum</em> infection</td>
<td>79</td>
<td>77</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Single <em>P. malariae</em> infection</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Single <em>P. ovale</em> infection</td>
<td>10</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>1†</td>
</tr>
<tr>
<td>Single <em>P. vivax</em> infection</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Mixed infection</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1‡</td>
</tr>
<tr>
<td>Total</td>
<td>102</td>
<td>81</td>
<td>4</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

*Plasmodium falciparum* × *P. ovale.*
† *P. ovale* × *P. falciparum.*
‡ *P. falciparum* × *P. vivax* × *P. ovale* (diagnosed by thin blood smear as a *P. ovale* × *P. malariae* infection).

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### Table 3

Profiles of 10 immigrants with a positive polymerase chain reaction (PCR) result*

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>PCR result</th>
<th>Thin smear result</th>
<th>IF titer</th>
<th>Origin of immigrant</th>
<th>Time interval†</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>21</td>
<td><em>P.f.</em></td>
<td>Neg.</td>
<td>200</td>
<td>Congo</td>
<td>15 days</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>23</td>
<td><em>P.f.</em></td>
<td>Neg.</td>
<td>800</td>
<td>Cameroon</td>
<td>NA</td>
<td>Healthy</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>26</td>
<td><em>P.f.</em></td>
<td>Neg.</td>
<td>1,600</td>
<td>Cameroon</td>
<td>18 months</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>3</td>
<td><em>P.f.</em></td>
<td>Neg.</td>
<td>100</td>
<td>Côte d'Ivoire</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>5</td>
<td><em>P.f.</em></td>
<td>&lt; 100</td>
<td>100</td>
<td>Gabon</td>
<td>60 days</td>
<td>Healthy</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>25</td>
<td><em>P.f.</em></td>
<td>&lt; 100</td>
<td>100</td>
<td>Gabon</td>
<td>3 years</td>
<td>Healthy</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>37</td>
<td><em>P.f.</em></td>
<td>Neg.</td>
<td>100</td>
<td>Benin</td>
<td>5 years</td>
<td>Healthy</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>17</td>
<td><em>P.f.</em></td>
<td>NA</td>
<td>400</td>
<td>Angola</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>40</td>
<td><em>P.m.</em></td>
<td>Neg.</td>
<td>200</td>
<td>Cameroon</td>
<td>6 years</td>
<td>NA</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>40</td>
<td><em>P.v.</em></td>
<td>NA</td>
<td>200</td>
<td>Djibouti</td>
<td>6 days</td>
<td>NA</td>
</tr>
</tbody>
</table>

*IF = immunofluorescence; *P.f.* = *Plasmodium falciparum*; Neg. = negative; NA = not available; *P.m.* = *P. malariae*; *P.v.* = *P. vivax*.
† Between arrival in France and the laboratory investigation.
ROUTINE PCR DIAGNOSIS OF IMPORTED MALARIA

Table 4
Profiles of five travelers with a positive polymerase chain reaction (PCR) result*

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>PCR result</th>
<th>Thin smear result</th>
<th>Country returning from</th>
<th>Time interval</th>
<th>Chemoprophylaxis</th>
<th>Antimalarial treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>35</td>
<td>P.f.</td>
<td>Neg.</td>
<td>Senegal</td>
<td>0</td>
<td>C + P</td>
<td>C + P continued</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>36</td>
<td>P.f.</td>
<td>Neg.</td>
<td>Indonesia</td>
<td>0</td>
<td>C + P</td>
<td>C + P continued</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>49</td>
<td>P.f.</td>
<td>Neg.</td>
<td>Angola</td>
<td>5 days</td>
<td>Mefloquine</td>
<td>Mefloquine continued</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>23</td>
<td>P.m.</td>
<td>P.m.</td>
<td>Zaïre</td>
<td>48 days</td>
<td>Mefloquine</td>
<td>Chloroquine</td>
<td>Healthy</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>29</td>
<td>P.o.</td>
<td>Neg.</td>
<td>Côte d’Ivoire</td>
<td>3 months</td>
<td>C + P</td>
<td>None</td>
<td>NA</td>
</tr>
</tbody>
</table>

* P.f. = Plasmodium falciparum; Neg. = negative; C + P = chloroquine + proguanil; NA = not available; P.m. = P. malariae; P.o. = P. ovale.
† Between arrival in France and the laboratory investigation.

Table 4. Immunodiagnostics was not performed on these patients. Further testing of their blood samples by thin smear examination found a P. malariae infection in only one patient. The three positive Pf PCR results among the travelers who had chemoprophylactic treatment were especially interesting. If these diagnoses be considered correct, the healthy outcome of these patients, in spite of the lack of any curative treatment, demonstrated the efficacy of chemoprophylaxis.

The three patients in group IV (P. falciparum) had no medical data available. A false-positive due to laboratory contamination can be excluded since all the control samples were negative.

Concerning species identification, data from Table 2 show that the PCR and thin blood smear results were consistent, apart from the differentiation of P. vivax and P. ovale infections. Both species are uncommon (2.64% of 529 samples) and a differential diagnosis when only young trophozoites were present was difficult, and could explain such erroneous optical diagnoses. Moreover, the PCR made a decisive contribution in the diagnosis of mixed infections, which are often misdiagnosed due to low parasitemia and difficult species identification. 25

Finally, the PCR appears to be a costly but efficient method for the diagnosis of malarial infection. If strict technical instructions were followed, daily use was possible without any major pitfalls. From this study, the PCR was found to be more efficient than optical methods in the detection of long-standing infections in immigrants and for an accurate diagnosis of mixed infections. It may also be an early warning test in the event of an unsuccessful chemoprophylaxis, but this point requires further investigation.

The PCR procedure has been established in the Laboratory of Parasitology of the University Hospitals in Toulouse as a second-line diagnosis among patients presenting clinical symptoms consistent with malaria, but with a negative optical diagnosis, including QBC™ testing, or when a difficulty in species identification occurs. Under such conditions, the delay in the diagnosis, which cannot be less than six hours, and which extends to 24–48 hours when the request for PCR testing occurs over a weekend, seems to be acceptable.

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