Multiple malaria infection in a pregnant woman from Nigeria: detection by multiplex PCR

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INTRODUCTION

Worldwide, malaria is one of the most common human infections. Pregnancy reduces women’s immunity, making them particularly vulnerable to illness, severe anemia and death (Bouyou-Akotet et al., 2003). In pregnant women, malaria increases the risk of spontaneous abortion, stillbirth, premature delivery and low birth weight - a leading cause of child mortality. The pathogenesis of malaria is best understood for infection with *P. falciparum*. Several factors contribute to the severity of clinical disease. Infected erythrocytes have a unique ability to adhere to host endothelium, contributing to microvascular occlusion, metabolic derangement and acidosis. Where the parasite burden is high, this produces the clinical manifestations of severe malaria: acute respiratory distress syndrome, renal insufficiency and cerebral malaria (Cooke et al., 2000). *P. vivax*, *P. ovale* and *P. malariae* generally produce milder symptoms than *falciparum* malaria. In the case reported here, we used multiplex PCR to investigate suspected mixed infection in a pregnant woman from Nigeria. The results suggest that the method used is highly specific and can be very sensitive and that it has several advantages with respect to microscopy and RDTs.

CASE REPORT

Clinical history and laboratory findings

A 34-year-old Nigerian woman was referred to the hospital with fever. The patient was in the 37th week of pregnancy. Ten days earlier she had returned to Italy after a 20 day trip to Nigeria. She had not used chemical prophylaxis against malaria. The patient’s body temperature at presentation was 39°C. She was vomiting and had diarrhea. A routine blood count revealed severe anemia (he-
moglobin 7.0 g/dl) and thrombocytopenia (58000/µl); values for leucopenia (2660/µl), lactate dehydrogenase (700 IU/L), γ-globuline (19%), conjugated bilirubin (8.4 mg/dl), and C-reactive protein (183 mg/l) were all above normal; albumin was low (49.2%). Other hematological and biochemical profiles were within normal limits. An assay for Plasmodium lactate dehydrogenase (OptiMal® Rapid Malaria Test, DiaMed, Morat Switzerland) detected P. falciparum. Thick and thin blood films were stained with Giemsa and read for malaria parasites following a standard, quality-controlled procedure (Alonso et al., 1994). The recorded parasitemia (density of asexual Plasmodium parasites in the blood) was 3,000 parasites/µl. Under the microscope, we observed that several red blood cells contained double rings, others contained single rings. Ring forms presented morphological characteristics of P. falciparum and probably of other Plasmodium species. To confirm our suspicion of a mixed infection, we decided to perform a molecular diagnosis.

**Molecular diagnosis**

DNA was extracted from 200 µl of the blood sample using the High Pure Template Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer’s instructions. Purified DNA template were used for amplification in a DNA thermal cycler using a species-specifics primers set as described elsewhere to perform nested-PCR (Snounou et al., 1993). Two distinct multiplex PCRs were used to test DNA samples for the presence of plasmodium infection: one multiplex was used to test for P. falciparum and P. vivax; another for P. malariae and P. ovale.

Each assay used 10 microliters of DNA. The assays were performed on a GeneAmp PCR system 2400 (Perkin-Elmer, Cetus). In each case, the reaction used 20 pmol of each primer, deoxyribonucleoside triphosphates (200 mM), 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.0), 50 mM KCl and 2.5 U of Hot Start Taq DNA Polimerase (Quiagen S.p.a., Milan, Italy) in a final volume of 50 µl. The amplification profile consisted of initial denaturation at 95°C for 15 minutes followed by 40 cycles of denaturation (94°C for 30 seconds), annealing (58°C for 1 minute) and extension (72°C for 1 minute). Amplicons were detected by electrophoresis of 20 µl of amplified products in a 2.5 % agarose gel (Eurobio) and visualized by ethidium bromide staining under UV light transilluminator Fluor-S™ multilmager (Bio-Rad). Molecular sizes of amplicons were determined by comparison with a commercial DNA molecular size marker (molecular weight marker VIII, Roche Diagnostics S.p.A. Monza. MI). The size of amplification products (205 bp for P. falciparum, 120 bp for P. vivax, 144 bp for P. malariae, and 800 bp for P. ovale), conformed to predictions (Figure 1). To assess the detection sensitivity, specific PCR products for each target sequence were serially diluted, cloned into pCR2.1 vector (Invitrogen, San Diego, CA) and amplified by multiplex PCR. Amplification of cloned sequences showed detection limits of 10 to 100 copies. Procedures to prevent specimen contamination and PCR carryover were rigorously observed at every stage. There were no indications of cross-reactivity or non-specific amplification.

Gel-electrophoresis analysis of amplification products showed one product (from P. falciparum) of 205 bp and one (from P. vivax) of 120 bp. These findings confirmed the presence of a mixed infection and allowed us to identify the second infecting species. Results obtained by multiplex PCR and by nested-PCR were in agreement.
DISCUSSION

Today, the most practical and reliable means for detecting parasites in blood samples is to examine stained blood smears under the microscope (Milne et al., 1994; Warhurst et al., 1996). The technique is cheap and easy to perform, and it provides, in skilled hands, effective quantification of infecting species that cause malaria in humans. However, it also has well-documented limitations: in particular it is relatively time-consuming and relatively ineffective in identifying parasites which are present only at low levels in the blood (Iqbal et al., 1999).

At times, the results can be difficult to interpret. In the case reported here, the presence of double rings bicastionate in red blood cells and high parasitemia allowed us to diagnose the presence of falciparum malaria. However, we found it difficult to identify all forms observed through the microscope, and we decided to perform a molecular diagnosis to confirm the possibility of a mixed infection.

The OptiMal® Rapid Malaria Test distinguishes infections with falciparum and non-falciparum parasites (P. vivax, P. ovale, P. malariae). However, it is unable to detect other Plasmodium species in samples that are positive for P. falciparum. So it is unsuitable for cases of mixed infection. Given these limitations we decided to use multiplex PCR to investigate multiple infections of Plasmodium in a single step.

In this case, the main advantages of multiplex with respect to nested-PCR are that:

1. multiplex PCR is far less labor-intensive (there is only one PCR step, compared to a least two in nested PCR);
2. the risk of contamination is minimal (there is no need to transfer amplified template from the primary to the secondary amplification reaction);
3. the result of the assay can be obtained in only 3 h. In summary, our case suggests that multiplex PCR assay offers a practical and clinically acceptable alternative to nested-PCR for rapid and accurate molecular diagnosis of patients presenting with symptoms of malaria. The method presented here provides one-step identification of the species involved in mixed infections and is more sensitive than microscopy in detecting parasites present at high densities in the blood.

REFERENCES


