NEW MICROBIOLOGICA, **31**, 565-567, 2008

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

Rosalia Graffeo[1], Luca Masucci[1], Francesca Bugli[1], Fernando Damiano[2], Carmen Pinnetti[2], Giovanni Federico[2], Giovanni Fadda[1]

[1]Institute of Microbiology;
[2]Institute of Clinical Infectious Disease, Catholic University of the Sacred Heart, Rome, Italy

SUMMARY

For the last 100 years, diagnosis of malaria has been based on examination of Giemsa-stained thick and thin blood films under the microscope. This is a time-consuming procedure which often fails to correctly diagnose the infecting species - especially when carried out by inexperienced technicians or when blood levels of parasite are low. Rapid Diagnostic Tests (RDTs) for antigen detection can distinguish between *Plasmodium falciparum* and *Plasmodium vivax* but cannot identify the species present in mixed infections. 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 RTDs.

KEY WORDS: Plasmodium, Molecular detection

Received April 28, 2008 *Accepted* July 09, 2008

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 for to investigate a suspected case of multiple Plasmodium infection.

Corresponding author
Dott. Rosalia Graffeo
Istituto di Microbiologia
Università Cattolica del Sacro Cuore
Policlinico "Agostino Gemelli"
Largo Agostino Gemelli, 8 - 00168 Rome, Italy
E-mail: liagrafit@yahoo.it

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 specie-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, deoxynucleoside triphosphates (200 mM), 1.5 mM $MgCl_2$, 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.

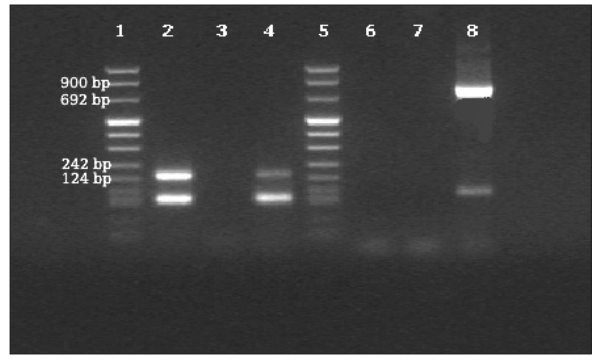

FIGURE 1 - *Detection of PCR-amplified Plasmodium DNA in agarose gel. Lane 1: DNA Molecular Markers; lane 2: positive sample; lane 3: negative control; lane 4: positive control; lane 5: DNA Molecular Markers; lane 6: negative sample; lane 7: negative control; lane 8: positive control. (From lane 2 to 4 multiplex - PCR Plasmodium falciparum 205 pb/Plasmodium vivax 120 pb; from lane 6 to 8 multiplex - PCR Plasmodium malariae 144 pb/Plasmodium ovale 800 pb).*

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 bicastonate 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

Alonso P.L., Smith T., Schellenberg Jr., Masanja H., Mwankusye S., Urassa H., Bastos De Azevedo I., Chongela I., Kobero S., Menendez C., et al. (1994). Randomised trial of efficacy of SP166 vaccine against *Plasmodium falciparum* in children in southern Tanzania. *Lancet*. **344** (8931), 1175-1181.

Cooke B.M, Wahlgren M., Coppel R.L.L. (2000). Falciparum malaria: sticking up, standing out and out-standing. *Parasitol Today*. **16**, 416-20.

Iqbal J., Sher A, Hira P.R., Al-Owaish R. (1999). Comparison of the OptiMal test with PCR for diagnosis of malaria in immigrants. *J. Clin. Microbiol*. **37**, 3644-3646.

Bouyou-Akotet M.K., Ionete-Collard D.E., Mabika-Manfoumbi M., Kendjo E., Matsiegui P.-B., Mavoungou E., Kombila M. (2003). Prevalence of *Plasmodium falciparum* infection in pregnant women in Gabon. *Malaria Journal*. **2**, 18.

Milne L.M., Kyi M.S., Chiodini P.L., Warhurst D.C. (1994). Accuracy of routine laboratory diagnosis of malaria in the United Kingdom. *J. Clin. Pathol.* **47**, 740-742.

Snounou G., Viriyakosol S., Zhu X.P., Jarra W., Pinheiro L., Do Rosario V.E., Thaithong S., Brown K.N. (1993). High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol. Biochem. Parasitol.* **61**, 315-20.

Warhurst D.C., Williams J.E. (1996). Laboratory diagnosis of malaria: ACO broadsheet no. 148. *J. Clin. Pathol.* **49**, 533-538.