The diagnosis of malaria and identification of *Plasmodium* species by polymerase chain reaction in Turkey

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Abstract

More than half of the world’s population is exposed to malaria in approximately 100 countries. Rapid diagnosis and correct treatment of cases are the main objectives of control programs in malaria endemic areas. We have developed a PCR method to determine the presence of *Plasmodium* DNA in blood. The method can also identify the species of the *Plasmodium* by restriction enzyme analysis of the amplified product. We evaluated the performance of this method in the diagnosis of malaria suspected cases in Turkey by comparing to microscopy of the blood smears: blood samples were obtained from 114 patients with malaria symptoms, including fever and/or chills lasting for several days, before starting treatment. Thin and thick blood smears were prepared immediately in the region of specimen collection. After isolation of DNA from blood samples, DNA was amplified by PCR and digested by restriction enzyme *Alu*I. The obtained fragments were analyzed by agarose gel electrophoresis. The number of parasites in the thick and thin smears of the blood samples was evaluated microscopically after staining by Giemsa and results were compared by PCR results. Among 114 *Plasmodium* positive cases detected by microscopy, 100 were also detected by PCR. There were 14 false negatives and no false positive by PCR. Compared to microscopy, the sensitivity, specificity and Positive Predictive Value (PPV) of PCR were determined as 76%, 100% and 100%, respectively.

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1. Introduction

Malaria is one of the most important parasitic diseases especially in tropical and subtropical areas such as Turkey. Four species of *Plasmodium* cause human malaria. Among these, *Plasmodium falciparum* is responsible for most of the mortality. *Plasmodium vivax* causes considerable morbidity and *P. malariae* and *P. ovale*, are less prevalent around the world [1–3]. The conventional method for the diagnosis of malaria has relied on the microscopic examination of Giemsa-stained blood smears. It is difficult to identify mixed *P. vivax* and *P. falciparum* infections, and requires a skilled microscopist when very low parasitemias are encountered, a situation frequently found with immune populations or when drugs are taken at subcurative levels [4,5]. We aimed to develop a PCR based methodology to detect plasmodium species in blood and to evaluate the diagnostic performance of PCR in the diagnosis of malaria-suspected cases in Turkey.

2. Materials and methods

2.1. Study area, subjects and blood sample collection

The malaria endemic areas of Turkey are located in the South-eastern Anatolia Region of Turkey. The weather of this
part of Turkey is generally warm and humid. These areas contain more than 95% of the total malaria cases in the whole country. Sanlıurfa is the most important area with more than 65% of all cases. *P. vivax* is the species responsible of almost all cases of malaria in Turkey. This study was conducted in Siverek, a city close to Sanlıurfa which is located in the Southeastern Anatolia Region of Turkey. Permissions for testing their blood sample were obtained from patients who admitted to the Siverek Malaria Eradication Centre. The majority of the blood samples were collected from individuals living in rural areas. A total of 114 whole-blood samples were obtained from patients with malaria-like symptoms including fever and/or chills of several days duration and who did not take any antimalarial drugs. A completed questionnaire was obtained from each patient. Blood samples which were obtained from 50 healthy individuals with approximately the same male to female ratio and the same range of age, with no history of malaria infection, were used as negative controls.

Two millilitres of venous blood was drawn into 1% EDTA-coated syringes, distributed into sterile test tubes, and placed immediately on ice. Thin and thick blood smears were prepared on the site at the time of specimen collection. The whole-blood samples were stored at −80 °C until extraction of DNA.

2.2. Microscopic determination of the parasite, and counting the parasitemia

All blood smears were stained with Giemsa and initially examined at the Siverek Malaria Eradication Centre by a malaria expert. They were then re-examined independently by two skilled microscopists. A smear was considered negative when no parasites were detected in the total area where 200 WBCs were observed by either of the microscopists [6].

Parasite density (parasites/μl blood) was calculated for each positive thick smear by assuming 8000 WBCs/μl blood. Thus, parasites in thick-smear fields were counted until 200 WBCs had been observed, and the parasite count was then multiplied by 40 to give the number of parasites per millilitre of blood. This method does not take the loss of parasites into account when preparing the thick smear and it produces an underestimate, but the “standard” white blood cell value which is probably too high in patients with malaria helps to cancel out this error [7].

2.3. DNA extraction and PCR

Extraction of parasite DNA from blood sample was carried out by using “Master Pure TM DNA Purification Kit for Blood” (Catalog Number: MG71100, Epicentre Biotechnologies, Madison, WI, USA) as described by manufacturers. Red Cell Lyses solution was added to the tubes of blood sample kept at room temperature for 10 min and mixed occasionally. Samples were centrifuged and then supernatant was discarded. Tissue Cell Lysis solution was added to the precipitate and mixed by pipetting. The samples were transferred on ice and kept for 3–5 min and centrifuged. The supernatant including DNA was transferred into a clean and sterile tube, isopropyl alcohol was added and then centrifuged to precipitate. The supernatant was discarded carefully and the precipitate was dried. Precipitated DNA was dissolved by 35 μl TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) and kept at −80 °C until used.

2.4. DNA amplification of Plasmodium by PCR

All PCR reactions were carried out with a mixture of 50 μl and contained: 27 μl H2O, 5.2 μl PCR 10x buffer, 5 μl 10x “Enhancer”, 5 μl MgCl2 (25 mM), 4 μl dNTP (0.2 mM each), 5 μl primer plasmodium F (10 pM/μl) (5′ TTTAAATTGTGCGAGTTAAAAACG 3′), 5 μl primer plasmodium R (10 pM/μl) (5′ CCAGACAATCATATCAGC 3′) [8], 0.25 μl Taq Polymerase (5 U/μl) (Epicentre Technologies). These primers amplify the 803 bp region of *P. vivax*, 808 bp region of *P. ovale* and 858 bp region of *P. falciparum*.

PCR was performed in a PTC-100 thermal cycler (MJ Research Inc.). PCR products were analyzed by electrophoresis using 2% agarose gels. RFLP Analysis: positive PCR products were cut with *Alu*I enzyme, analyzed by electrophoresis using 2% agarose gels. The bands were visualized on a UV transilluminator after staining with ethidium bromide. *Alu*I enzymes cut the 803 bp DNA product amplified from *P. vivax* into 335, 260, 73, 70 and 65 bp fragments. The 808 bp product amplified from *P. ovale* is cut by *Alu*I into 337, 333, 73 and 65 bp fragments and the 858 bp product amplified from *P. falciparum* is cut into 350, 301, 73, 65, 45 and 24 bp fragments. The small fragments are hard to visualize in the gel; however, the two largest restriction fragments obtained from amplified DNA of plasmodia enable differentiating the species (Fig. 2).

3. Results

Forty-four cases were diagnosed as malaria with PCR using *plasmodium* F and *plasmodium* R primers (Fig. 1) and forty-four cases were diagnosed as *P. vivax* malaria with restriction fragment length polymorphism (RFLP) method using *Alu*I enzyme (Fig. 2).

Fifty-eight of 114 cases were diagnosed as malaria with PCR using *plasmodium* F and *plasmodium* R primers (Fig. 1) and forty-four cases were diagnosed as *P. vivax* malaria with restriction fragment length polymorphism (RFLP) method using *Alu*I enzyme (Fig. 2).
blood smears. The diagnostic performance of PCR is shown in Table 1. Plasmodium DNA was not detected in any of the 50 healthy controls.

4. Discussion

Although microscopic detection of parasites on Giemsa-stained blood smears has been the reference standard method for malaria diagnosis in laboratories for more than a century, it is an imperfect standard, highly dependent on the technical expertise of the microscopist. Microscopic diagnosis often leads to a delay in making proper decision on anti-malarial drug treatment. Also, this method can sometimes be misleading in identifying Plasmodium species especially in cases with low level of parasitemia, a mixed parasite infection, or when modified by anti-malarial drug treatment [4,5,9]. As molecular methods developed and became more applicable in routine diagnosis, the additional advantages of these, like providing information about drug-resistance and genetic diversity of malaria parasites led scientists to apply these methods more commonly. Snounou et al. have reported that PCR has proven to be more sensitive and accurate than routine diagnostic microscopy in detection and identification of the parasites [5]. Relf at al. have reported that it is difficult to evaluate the specificity of PCR for the identification of P. falciparum or P. vivax in a single sample with very low plasmodium concentration; however, the evaluation of PCR method together with the thin smears can be accepted as the gold standard. It was also reported that various problems could be encountered while collecting samples from the area and in the transportation of the samples and in the process of plasmodium DNA extraction from the samples. DNA amplification can be inhibited with the ions existing in the whole blood [9].

Among 114 cases which had malaria symptoms, P. vivax was detected by evaluating thick blood smears in 58 and by PCR in only 44. The other plasmodium species were not detected by either of the methods in patient samples. Fourteen of the 114 cases were determined as false negative by PCR and no false positive result was obtained. The sensitivity, specificity and Positive Predictive Value (PPV) were determined as 76%, 100%, and 100%, respectively. The increase in the sensitivity and Negative Predictive Value (NPV) of PCR in cases with high load of plasmodium attracts attention (shown in table). The sensitivity and NPV were determined as 65% and 85% respectively in cases with parasitemia level less than 500/μl blood and as 92% and 98% respectively in cases with parasitemia level more than 5000/μl blood. A discordant case in the second group with 26,000 plasmodium was not detected with PCR. The reamplification and purification of DNA for this case did not change the result. All of the control samples obtained from healthy patients were negative for plasmodium DNA by PCR.

Koltaş et al. in a study held at Çukurova region of Turkey, reported that 20 (40%) of the 50 cases which were diagnosed as malaria according to clinical symptoms and positive blood smears, were all determined as positive by PCR. The blood samples were collected on filter papers in their study. For this study we collected samples into microcentrifuge tubes containing 0.2 ml 1% EDTA. We detected P. vivax DNA by PCR in 44 (76%) of 58 cases which were diagnosed as malaria according to the clinical symptoms and positive blood smears. Preservation of DNA in liquid form may not be as good as preserving it dry on filter papers which may explain our inability of detecting plasmodium DNA in 14 samples that contained plasmodia [10].

Comparing to microscopy, Laseron et al. determined the sensitivity and specificity of DNA probe/PCR method as 62% and 96%, respectively, in the diagnoses of P. falciparum [11]. Prompt and accurate diagnosis is the key to effective disease management and one of the main interventions of the Global Malaria Control Strategy. Microscopy offers many advantages such as high sensitivity, ability to provide additional information (type of the species, circulating stage and parasite density), and low cost. Microscopy suffers from three main disadvantages: being labour-intensive, time-consuming and requiring experienced microscopists [12].

WHO reported that PCR is more sensitive and specific than all other techniques. It is, however, a lengthy procedure that requires specialized and costly equipment and reagents, as well as laboratory conditions that are often not available in the field [13].

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Table 1: The diagnostic performance of PCR in the diagnosis of malaria

<table>
<thead>
<tr>
<th>Parasites/μl</th>
<th>PCR positive samples</th>
<th>Blood smear positive samples</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value (PPV)</th>
<th>Negative Predictive Value (NPV)</th>
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<tr>
<td>1 – 500</td>
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<td>26</td>
<td>65</td>
<td>100</td>
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<td>85</td>
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<td>5</td>
<td>60</td>
<td>100</td>
<td>100</td>
<td>96</td>
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<tr>
<td>1001 – 5000</td>
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<td>14</td>
<td>86</td>
<td>100</td>
<td>100</td>
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<tr>
<td>5001 – 10,000</td>
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<td>13</td>
<td>92</td>
<td>100</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>58</td>
<td>76</td>
<td>100</td>
<td>100</td>
<td>78</td>
</tr>
</tbody>
</table>

Fig. 2. Typical Alu I restriction fragments obtained from a PCR product amplified from a patient infected with Plasmodium vivax (M: Molecular weight marker, ΦX174 DNA/HaeIII digest; 1: PCR product digested with Alu I; 2: undigested PCR product).
Kho et al. have described the multiplex PCR for differential diagnosis between *P. falciparum* and *P. vivax* infections by targeting the 18S rRNA gene with a set of primer combinations. This method is highly useful as the differential diagnosis in areas, where malaria has recently re-emerged and fully trained microscopists are limited in number [14].

Rubio et al. recommended that this molecular method showed more sensitivity and specificity than microscopy, detecting 12.4% more positive samples than microscopy and 13% of mixed infections were undetectable by Giemsa stain. The use of a seminested multiplex PCR permitted confirmation of the origins of the infections and the *Plasmodium* species involved and confirmation of the effectiveness of drug treatment. Also, PCR allowed the detection of the presence in Spain of primaquine-tolerant *P. vivax* strains from west-central Africa [15].

Nested PCR has been reported to be more useful than conventional microscopy in selected cases for the diagnosis of low-level parasitemia and correct diagnosis of malaria parasite species and correct treatment [16].

In conclusion, the method developed in this first study using PCR and restriction enzyme analysis is an effective way to diagnose and identify the species of plasmodia in blood samples. The sensitivity of our method, which turned out to be less than microscopy, may be increased by obtaining samples onto filter papers instead of obtaining into tubes and storing in liquid form.

References