Detection of malaria infection in blood transfusion: a comparative study among real-time PCR, rapid diagnostic test and microscopy

Sensitivity of Malaria detection methods in blood transfusion

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Abstract The transmission of malaria by blood transfusion was one of the first transfusion-transmitted infections recorded in the world. Transfusion-transmitted malaria may lead to serious problems because infection with Plasmodium falciparum may cause rapidly fatal death. This study aimed to compare real-time polymerase chain reaction (real-time PCR) with rapid diagnostic test (RDT) and light microscopy for the detection of Plasmodium spp. in blood transfusion, both in endemic and non-endemic areas of malaria disease in Iran. Two sets of 50 blood samples were randomly collected. One set was taken from blood samples donated in blood bank of Bandar Abbas, a city located in a malarious-endemic area, and the other set from Tehran, a non-endemic one. Light microscopic examination on both thin and thick smears, RDTs, and real-time PCR were performed on the blood samples and the results were compared. Thin and thick light microscopic examinations of all samples as well as RDT results were negative for Plasmodium spp. Two blood samples from endemic area were positive only with real-time PCR. It seems that real-time PCR as a highly sensitive method can be helpful for the confirmation of malaria infection in different units of blood transfusion organization especially in malaria-endemic areas where the majority of donors may be potentially infected with malaria parasites.

Introduction

Malaria is known as the most important transfusion-transmitted disease worldwide (Kitchen and Chiodini 2006). Based on the latest report of World Health Organization, 243 million cases were reported from 109 countries with 863,000 death in 2008 (WHO 2009). Malaria is one of the public health problems in Iran also, 80% of cases has been reported from south and southeastern areas of the county, with a total population of 3,000,000 people at risk of the disease transmission. Furthermore, the imported cases from Afghanistan and Pakistan to the areas with potential transmission, makes the problem more difficult to control (Raeisi et al. 2009).

Although malaria transmission occurs principally through mosquito bites, there have been reports of transfusion-transmitted malaria since the beginning of the twentieth century. The parasites load in infected donors may be very low; therefore, no clinical symptoms may be observed and
Plasmodium species may live in the donors for years. As the agents may live long in the body of donors, the blood from donors should be examined thoroughly for agents. Any blood component containing erythrocytes can harbor viable parasites. Although whole blood and red blood cells (RBCs) concentrates represent the most common source of transfusion-transmitted malaria, cases involving platelets, leukocytes, fresh frozen plasma, and frozen RBCs have all been reported also (Elghouzzi et al. 2008).

Malaria screening in blood banks is based on the selection of donors in respect to possible associated risks with travel or residence, clinical evidence, and/or inaccurate diagnostic methods, thereby increasing the probability of transfusion-transmitted infection.

Laboratorial diagnoses, in general, are made using the thick and thin blood film techniques. However, induced morphologic alterations can occur during the staining of slides, modifying the data of these species (Cavasini et al. 2000). Moreover, some parasitic development stages can lead to errors in diagnosis, even with extremely experienced microscopists. In experienced hands, a sensitiveness between five and 50 parasites per micro liter can be achieved, but most laboratories achieve a lower sensitivity of around 500 parasites per micro liter (Seed et al. 2005). In comparison, polymerase chain reaction (PCR) is a highly sensitive and specific method and has shown to be efficient in the diagnosis of human malaria parasites particularly, identifying high prevalence of mixed infections (Postigo et al. 1998). However, PCR have its limitations including its high cost and the necessity of adequate equipment and trained technicians. The suitability of each method for donor screening needs to consider several key criteria including the prevalence of and immunity to malaria in the donor population, test sensitivity (particularly for P. falciparum), cost, reliability, speed, and complexity. One of the main considerations is test sensitivity because it has been shown that as few as ten parasites per unit of RBCs are sufficient to transmit infection (Boyd 1949).

Bandar Abbas, the main seaport of southern Iran, is an economic malaria area with high number of passengers from different parts of Iran as well as other countries. Malaria is endemic in the Iran and local transmission occurs normally (Hanafi-Bojd et al. 2010).

The aim of this study was to compare the agreement rate of three distinct diagnostic methods (PCR, rapid diagnostic test and blood smear examination) for malaria parasites detection in blood donor samples of Bandar Abbas as an endemic area and Tehran, the capital city of Iran, as a non-endemic location.

**Materials and methods**

**Study area** Bandar Abbas is the capital city of Hormozgan province, situated in the southern part of Iran. It is located between 54° 53′–56° 03′ E and 26° 53′–27° 31′ N on flat ground with an average altitude of 9 m above sea level. The city has a hot and humid climate. Maximum temperature in summers can reach up to 49°C while in winters the minimum temperature drops to about 5°C, and the mean annual relative humidity is about 65%, rises up to 95% in summer (www.weather.ir). Total population of this city currently is more than 520,000 people. About 77% of population of this district is living in urban area and 23% in rural area. Bandar Abbas was selected as the endemic area for malaria in our study.

Tehran is the capital city of Iran and Tehran province with a population of more than 8,500,000 people. Tehran features a semi-arid, continental climate. The hottest month is July (mean temperature: 26–36°C) and the coldest is January (mean temperature: −1 to 8°C). This city is considered as a free-malaria area in our study (www.weather.ir).

**Study design and sample collection techniques** Using statistical formula, P value of 5% and the agreement rate of 80%, the sample size (n) was calculated as at least 96, so we collected 100 blood samples. Half of this samples were obtained from Bandar Abbas, the remaining from Tehran blood banks.

**Laboratory techniques** Blood samples were taken randomly from referrers to blood banks after having completed data forms for history of malaria infection, or fever and chills and also anti-malaria drug consumption. Then, two blood sample tubes including anti-clotting EDTA were filled from their vein blood. Tubes were shaken and immediately transferred to −20°C freezer for real-time PCR and Dipstick tests. Two thin and tick blood smears were also prepared for microscopic examination.

**Light microscopic examination** The smears were fixed by methanol and stained using Giemsa method. One and two hundred fields from thick and thin smears, respectively, were examined carefully (WHO 1991).

**Dipstick or rapid test (PLDH/HRP2)** We used the dipstick kits of Premier Medical Corporation Ltd. (Cat.No.116FRC 30). They are consisting of one strip covered by two monoclonal antibodies in two lines, one contains lactate dehydrogenase for detection of P. vivax, P. malariae, and P. ovale, and another contain antibody for histidine-rich protein II to detect P. falciparum (Gillet et al. 2010).
Real-time PCR DNA extraction from blood samples was conducted using Genet-Bio® (Cat No: K-2000) kit. Then DNA quality (the ratio of OD260/280) and quantity (OD260) was determined using Biophotometer instrument. Each DNA sample (50 ng) was added to real-time PCR tubes containing Syber Green master mix, specific primer set for either Plasmodium spp. or P. falciparum (Table 1) and diethyl pyrocarbonate-treated water. In addition to unknown blood samples, the DNA extracted from available samples of P. falciparum and P. vivax were used as positive controls. Following assay optimization using positive DNA controls and negative sample (no template control, NTC), all unknown samples of DNA were subjected to amplification using HRM6500 Corbette real-time PCR instrument. Finally, in addition to the analysis of data by Corbette software to determine the positive and negative DNA samples for parasite infection, the results of real-time PCR were further confirmed by agarose gel electrophoresis of amplified samples (Azizi et al. 2010) to observe single band of expected size of amplicon (Fig. 1).

Ethical approval There was no ethical problem because all samples were obtained from blood donors. Moreover, the results of examination were given to them, free of charge.

Data analysis Fisher test was used to compare age and sex distribution of samples in two blood banks of Tehran and Bandar Abbas. Analyses were done using SPSS (version 13.5; SPSS Inc, Chicago, IL, USA), with a probability ($P$) value of <0.05 as statistically significant.

Results

Most of volunteers were male (96%). Their age ranged from 19 to 72 years old with an average of 37±11.83. Using Fisher’s test, samples of Tehran and Bandar Abbas had no difference considering the age and sex ($P$>0.05).

All samples were negative by both microscopy and Dipstick methods, but we found two positive samples collected from Bandar Abbas as an endemic area of malaria in Iran using real-time PCR. After DNA extraction and real-time PCR using the designed primers for Plasmodium spp. (Table 1), agarose gel electrophoresis of some samples including the P. vivax and P. falciparum as positive controls confirmed the amplification of a single band with expected size (Fig. 1). The NTC and sample B2 showed no band indicating the lack of DNA contamination and negative blood infection, respectively. The samples B46 and B50 from endemic area showed the same band as positive controls with less intensity that indicates positive blood infection with low level of parasite’s DNA in these two blood samples.

The real-time PCR graph (Fig. 2) shows an increase in fluorescence of Syber Green dye during cycles of amplification using specific primer set for Plasmodium spp. in the two positive controls and to less extent the two blood samples from endemic area of Bandar Abbas (B46 and B50). On the other hand, the fluorescence intensity of Syber Green dye during cycles of amplification was much less using specific primer set for P. falciparum in the B46 and B50 samples and no amplification for P. vivax in comparison to the P. falciparum-positive DNA sample (Fig. 2). The NTC and all other 98 blood DNA samples showed no amplification with either Plasmodium spp. or P. falciparum primer sets under the same experimental conditions indicating the lack of DNA contamination and negative blood infection, respectively.

Discussion

Malaria, one of the most important parasitic diseases in under-developed countries, is a serious transfusion-transmitted infection ranked after viral hepatitis and HIV. The extensive use of blood and its products, and close contacts of human beings, enhanced the risk of transfusion-transmitted malaria. Plasmodium species can be transmitted by transfusion of cellular components in labile blood products, and unlikely by frozen/thawed therapeutic plasma. Live Plasmodiae can develop and survive for about 20 days at 4°C, the conditions used for banking RBC concentrates or whole blood (Bruce-Chwatt 1972). Asymptomatic carriers have potential role as the source of infection for Anopheles vectors as well as blood recipient. An experimental study with asymptomatic carriers found an

<table>
<thead>
<tr>
<th>Organism</th>
<th>Primer</th>
<th>T. annealing °C</th>
<th>PCR product (amplicon size) bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmodium spp</td>
<td>F: AACATGGCTATGACGGGTAACG</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>R: GCCGTGCTGCTTCACTTAGATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. falciparum</td>
<td>F: GGGTTTACAGCAAGATAGTATGG</td>
<td>62</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>R: CCTCCTCTCTATTTCGTTGTC</td>
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The infection rate of 1.2% for *Anopheles darlingi*, the main Brazilian vector (Alvez et al. 2005).

The first case of transfusion-transmitted malaria was reported in 1911 (Woolsey 1911). After that report, *P. vivax* was predominated until the 1950s when *P. malariae* replaced it as the most common causative species. However, in the 1970s and now an increasing proportion of cases due to *P. falciparum* are seen (Bruce-Chwatt 1972).

Test methods for malaria can be broadly classified into two categories: direct and indirect. Direct methods detect parasite or parasite subcomponents, examples of which include microscopic examination of stained slides, circulating parasite antigens (histidine-rich protein 2, plasmodial lactate dehydrogenase, or aldolase in Dipstick kits), or plasmodial DNA (PCR). Indirect methods detect host responses to infection; examples include antimalarial antibodies (indirect immunofluorescent antibody test or IFAT, enzyme immunoassay or EIA) and iron pigment detection (hemozoin; Seed et al. 2005).

Rechecking 74,420 negative blood smears during 2004–2008 in Bandar Abbas showed 0.15% positive (Hanafi-Bojd et al. 2010). The failure in detection of mixed infections by the thin and thick blood film methods make treatment difficult as it is species-specific. Microscopic diagnoses failed to detect low parasitemia in PCR-positive asymptomatic individuals living in endemic regions of Brazil (Scopel et al. 2004). Therefore, the optimum strategy for minimizing the risk of transfusion-transmitted malaria in endemic areas is a combination of appropriate donor selection together with donation screening using PCR and other laboratory methods (Fugikaha et al. 2007).

In the context of a 250-mL unit of RBCs, a test sensitivity of 0.00004 parasites per microliter of RBCs would be required to identify a potentially infectious donation. In respect of indirect tests, of which serology assays are by far the most common, sensitivity for low-titer antibodies apparent early in infection and specificity in low prevalence populations, as well as antibody recognition of all four species, are key considerations (Seed et al. 2005).

Malaria transmitted by transfusion may be lethal for blood receiver, if not detected on-time. Although in nearly all countries including Iran, they do not use the blood of donors with a history of malaria during 3 years before donation, asymptomatic carriers are a potential risk and blood screening should be carried out in blood banks (Edrissian 1985).

Swan et al. (2005) used real-time PCR for detection of malarious patients. They found an agreement of 86% between these methods with the standard microscopic examination of blood smears. Our study showed an agreement of 98% between these two methods. This higher rate may be partly due to better quality of microscopic...
examination in the studied blood banks. Vo et al. (2007) introduced real-time PCR as a good method for malaria detection in passengers to the endemic areas of the disease. Based on the study of Vo et al., 79 persons without any symptom of malaria were examined and plasmodial infection was found in seven and 16 blood samples using standard microscopic and real-time PCR methods, respectively.

In conclusion, if real-time PCR is optimized correctly, it can be used in blood banks of the endemic areas to detect asymptomatic carriers of malaria, although dipstick tests can facilitate rapid and correct diagnosis. Periodical training courses for technicians of blood banks, especially in non-endemic areas where they may not be so expert for malaria parasites detection may be helpful.

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References

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