Journal of Medical Sciences

ISSN 1682-4474

science alert

ANSInet
an open access publisher
http://ansinet.com

Research Paper

JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued eight times per year on paper and in electronic format.

For further information about this article or if you need reprints, please contact:

Abdolhossein Dalimi
Department of Parasitology,
Faculty of Medical Sciences,
Tarbiat Modares University,
P.O. Box 14115-111, Tehran,
Islamic Republic of Iran

J. Med. Sci., 7 (7): 1082-1087
1st October, 2007

Application of Real-Time PCR for Detection of *pfcrt* Single Nucleotide Polymorphisms in *Plasmodium falciparum* in Southeast Iran

[1]Jalousian Fatemeh, [1]Dalimi Abdolhossein, [2]Mirab Samiee Siamak, [1]Ghaffarifar Fatemeh, [3]Soleymanloo Faramarz and [3]Naghizadeh Ramin

The main objective of present study was to evaluate the efficiency of real-time PCR technique for detection of 72-76 *pfcrt* gene mutations. In this regard, by using hybridization probes technology on light cycler instrument, the point mutations have been successfully detected in 28 blood samples collected from falciparum malaria patients in Chabahar in Southeast Iran. Our data showed *pfcrt* K76T mutation is present in 99% of samples. Three samples (10.7%) showed deletion in amino acid located in position 75, Asparagine, one of them was located at CVMK and two at SVMT alleles. Sequencing analysis of these samples was the same as real-time PCR result. Five samples were found with multi-clonal population of *P. falciparum*, which identified by the presence of the two peaks simultaneously in melting curve analysis. Real time PCR is found a sophisticated technique that can distinguish these mutations reliably with acceptable speed, high accuracy, sensitivity and reproducibility.

Key words: *Plasmodium falciparum*, CQ resistance, *pfcrt* gene, single nucleotide polymorphisms, real-time PCR

[1]Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Islamic Republic of Iran
[2]Food and Drug Control Laboratories, Ministry of Health and Medical Education, Tehran, Iran
[3]Day General Hospital, Clinical Chemistry and Molecular Diagnosis Department, Tehran, Islamic Republic of Iran

ANSInet
Asian Network for Scientific Information

INTRODUCTION

Chloroquine is the cheapest and the most widely prescribed anti-malarial drug in almost all the endemic countries. Since the first report of chloroquine (CQ) resistant falciparum malaria in south-east Asia and South America, almost half a century ago, drug resistance in malaria is consider as a vitally important public health concern (Wongsrichanada *et al*., 2002). Understanding the mechanisms of such resistance is urgently essential in malaria endemic area. It could contribute to information-based policy decisions on future therapeutic options against malaria. Great progress has been made recently in studying the mechanisms of drug action and drug resistance in malaria parasites, particularly in *Plasmodium falciparum*. These efforts are highlighted by the demonstration of mutations in *P. falciparum* chloroquine-resistance transporter (*pfcrt*) gene. These mutations may severely limit the therapeutic options for the treatment of malaria. With ever-increasing failure rates associated with chloroquine treatment, attention has turned to the few alternatives, which include quinine and mefloquine in some part of the world (Sangster *et al*., 2002).

Point mutations in *pfcrt* gene have been shown to be associated with CQ resistance phenotype (Babiker *et al*., 2001). Now a day, polymorphisms in the *pfcrt* gene of the *P. f* genome are the focus of studies on the molecular basis of CQ resistance in endemic areas. The *pfcrt* gene is located on chromosome 7 and codes for *pfcrt*, a vacuolar membrane transporter protein (Wongsrichanada *et al*., 2002).

The several studies performed on isolates from Africa (Babiker *et al*., 2001; Basco and Ringwald, 2001; Djimde *et al*., 2001; Dorsey *et al*., 2001; Mayor *et al*., 2001), Asia (Pillai *et al*., 2001; Lim *et al*., 2003) and South America (Vieira *et al*., 2001) have shown that the K76T mutation is present in almost all CQR samples.

In Iran *P. falciparum* transmission mainly occurs in the South eastern of the country. Chabahar is a harbor located in South-east Iran, where more than 85% annual malaria cases occur. Some workers (Edrissian and Shahabi, 1985; Zakeri *et al*., 2002; Ursing *et al*., 2006) had reported CQ resistance (CQR) in endemic areas of Iran previously. In a study carried out by Ursing *et al*. (2006) in South-east of Iran, polymorphisms in the *pfcrt* and the *pfmdr1* genes were detected by PCR-RFLP and sequencing methods.

There are four basic methods for testing malaria for drug resistance; *in vivo* tests, *in vitro* tests, molecular characterization and animal models. Of these, only the first three are routinely done. For chloroquine molecular markers have been identified that confer resistance. Molecular techniques, such as Polymerase Chain Reaction (PCR) or gene sequencing can identify these markers in blood taken from malaria-infected patients. Real time PCR is a novel technique that recently has utilized successfully for drug sensitivity assessment (Cheesman *et al*., 2003; de Monbrison *et al*., 2003).

The main objective of present study was to evaluate the efficiency of real-time PCR technique for detection of 72-76 *pfcrt* gene mutations in blood samples taken from malaria patients in endemic area of South-east Iran.

MATERIALS AND METHODS

Blood samples: Twenty eight blood samples were collected from Iranian individuals, infected with *P. falciparum* in Chabahar. The infection was confirmed by microscopic examination of thick and thin smears. The samples were stored at -20°C until use.

The DNA was isolated from blood samples using QIAamp DNA minikit (Qiagen®, Germany) according to manufacturer's instructions.

***Plasmodium falciparum* reference strains:** Three *P. falciparum* strains selected as references. CQ-sensitive strain was 3D7 and CQ-resistant strains were W2 and 7G8. These strains were generously provided by Dr. de Monbrison from Claoude Bernard University, Lyon, France.

Detection of 72-76 *pfcrt* gene mutations: Detection of mutations was carried out with light cycler ™ using hybridization probes according to de Monbrison (de Monbrison *et al*., 2003) method.

For *pfcrt* mutations, an amplification primer iLC Labeled with Light Cycler™ Red 640 on the third base from the 3' end was used.

During amplification, extension of the primer iLC occurs, allowing hybridization to the 3'-FITC labeled sensor probes. FRET is occurred between the sensor probe and the whole PCR product working as anchor probe (de Monbrison *et al*., 2003).

A specific melting temperature is obtained for each genotype; a sensor probe spanning one mismatch could still hybridize to the target sequence but melted off at lower temperature than a sensor probe with a perfect match (de Monbrison *et al*., 2003).

Primers and probe were synthesized by TIBMOLBIOL (DNA Synthesis Service, Germany), to detect 72-76 mutation of *pfcrt* gene (Gen Bank: AF233068).

The primers and probe used for detection of *pfcrt* M741, N75E, K76T mutations were:

Primer F: 5'- CTTGTCTTGGTAAATGTGCTCA-3'
Sensor: 5'- TGTGTAATTGAAACAATTTTTGCTAA-3'
iLC: 5'- GTTACCAATTTTGTTTAAAGTTCT-3'

PCR amplification: For *pfcrt* codons, after an initial step at 95°C for 10 min, amplification was performed with 40 cycles of denaturation (95°C for 10 sec), annealing (45°C for 10 sec) and extension (65°C for 15 sec). The temperature transition rates were 20°C sec^{-1} for denaturation and annealing and 5°C sec^{-1} for extension. The melting curve program consisted of one cycle of 95°C for 2 sec, 32°C for 20 sec and heating at 75°C. The temperature change rates were 20°C sec^{-1} except for the final step which had a temperature transition rate at 0.2°C.

Reference samples have been included in each runs as references.

PCR product cloning: PCR product was purified using PCR purification kit for recovery of DNA fragments from agarose gel (Fermentas®, Lithuania), according to the manufacturer's recommendations. Purified PCR product was cloned in to PTZ57R/T Vector (Fermentas®, Lithuania).

The ligation product was transformed into *Escherichia coli*, TG1 strain, competent cells. Then, plasmids from positive colonies, white colony, were extracted for sequencing. Sequencing of both strands was done using Mb/puc primers, by MWG company.

Sequencing used as a gold standard to confirm the real-time PCR results.

Detection of multi clonal infection: In order to evaluate the capacity of the method to detect multi-clonal infection, 3D7 strain (has wild type allele in all codon positions) and W2 strain (has mutant type allele in all codon positions) were mixed in proportion of 1/10, 1/8, 1/5, ½, 4/5 and 9/10. Then melting curve analysis of mixed strains was used for the detection of mutation.

Evaluation of technique reproducibility: To evaluate technique reproducibility, melting temperature of reference strains was done in 4 independent assays.

RESULTS

The potential for detection of 72-76 point mutations in *pfcrt* gene using hybridization probes and LightCycler™ technology was evaluated with comparison of melting temperatures™ of blood samples and reference strains (Fig. 1).

3D7 strain showed wild *pfcrt* genotype for all codon positions, 72, 73, 74, 75, 76 and its melting temperature was 41.3±0.2°C (Fig. 1). Only one samples showed the Tm similar to 3D7.

W2 strain showed the highest melting temperature, 52.9±0.7°C and mutant *pfcrt* genotype for all codon positions (Fig. 1). Seven samples showed Tm like W2.

7G8 strain showed mutant *pfcrt* genotype for codon position 76 and wild *pfcrt* genotype for codons positions 74, 75, its melting temperature was 43.7±0.2°C (Fig. 1).

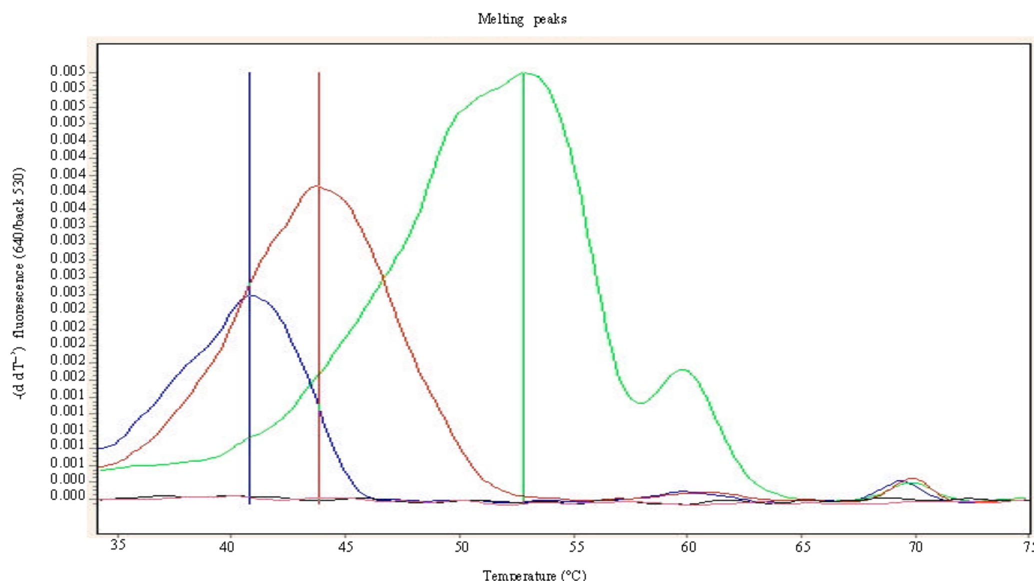

Fig. 1: Melting curve analysis of W2, 7G8 and 3D7 (reference strains) from right to left respectively

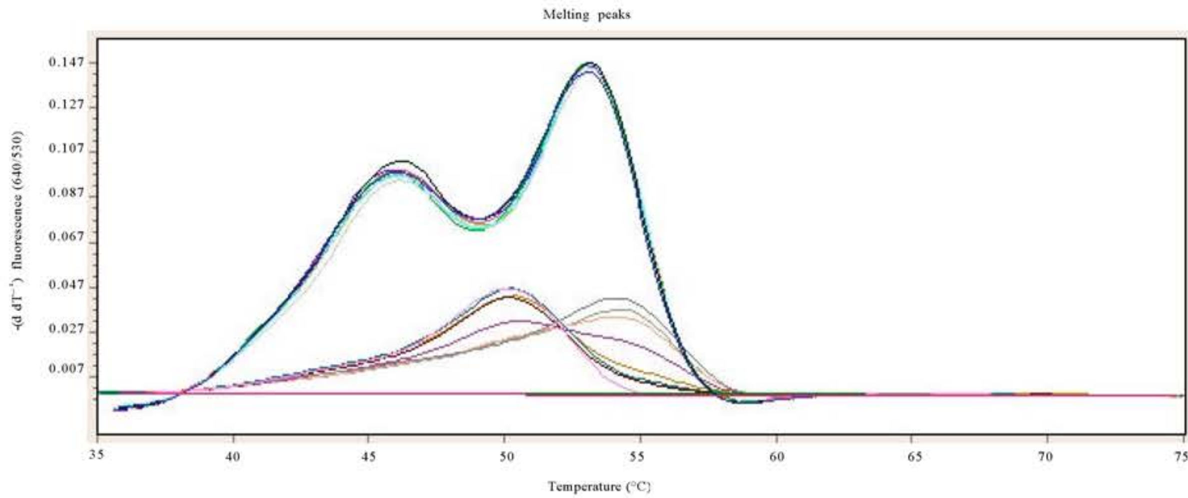

Fig. 2: Upper curves: Melting curve analysis of five samples which infected with multi-clonal population of *P. falciparum.* Lower curves: Mixed strains of 3D7 and W2 in proportion of 1/10, 1/8, 1/5, ½, 4/5 and 9/10. The curve with two wide peaks is belonging to proportion of ½ (50% 3D7 with 50% W2), each peak corresponding to the melting temperature of the reference strains

RiB_PRIMERF -- 27......121 of sequence

GAT AAT ATT TTT ATT TAT ATT TTA AGT ATT ATT TAT TTA AGT GTA
AGT --- A**C**A ATT TTT GCT AAA AGA ACT TTA AAC AAA ATT GGT
C72S 75 deletion K76T
AAC AAC AAT CA.

Fig. 3: Sequencing results represent the deletion of amino acid located in position 75, Asparagine, in addition to C72S and K76T mutations in SVMT allele

ZaB15_PRIMERF -- 27.....121 of sequence

GAT AAT ATT TTT ATT TAT ATT TTA AGT ATT ATT TAT TTA AGT GTA
TGT --- AAA ATT TTT GCT AAA AGA ACT TTA AAC AAA ATT
75 deletion K76
GGT AAC AAC AAT CA.

Fig. 4: Sequencing result represent only deletion of amino acid located in position 75, Asparagine in CVMK allele

Present data indicated that, all but one sample showed the *pfcrt* K76T mutation. Eleven samples showed similar Tm and sequencing analysis to 7G8.

Melting curve analysis of mix of 3D7 and W2 for the detection of M74I, N75E, K76T *pfcrt* mutations is shown in Fig. 2. Multi-clonal population was identified (wild-type and mutant-type alleles) by the presence of the two peaks simultaneously. Five (17.8%) samples showed two peaks simultaneously (Fig. 2). Each peak is corresponding to the melting temperature of specific strains.

Amino acid changes in *pfcrt* gene of *P. falciparum* found are shown in Table 1. According to the Table 1, mutation of C72S in SVMNT and SVMT alleles, mutation of M74I in CVIE allele, mutation of N75E in CVIET allele, mutation of K76T in CVMNT, SVMNT, CVIET, SVMT were observed. The rates of mutation in CVMNT, SVMNT, CVIET, SVMT and CVMK were 39.28, 25, 25, 7.14 and 3.57%, respectively.

Two deletions of Asparagine in position of 75 were found in sequence of SVMT and CVMT alleles (Fig. 3 and 4).

Table 1: Amino acid changes in different alleles of *pfcrt* gene of *P. falciparum* found in Chabahar, South-east of Iran

	Mutations				
Alleles	C72S	M74I	N75E	K76T	Mutation rate (%)
CVMNT	C	M	N	T	11 (39.28)
SVMNT	S	M	N	T	7 (25.00)
CVIET	C	I	E	T	7 (25.00)
SVMT	S	M	Deletion	T	2 (7.14)
CVMK	C	M	Deletion	K	1 (3.57)
Total	7	7	7	27	28

C: Cysteine, S: Serine, M: Methionine, I: Isoleucine, N: Asparaginee, E: Glutamic acid, T: Threonine, K: Lysine

DISCUSSION

One of the greatest challenges facing malaria control is the spread and intensification of parasite resistance to antimalarial drugs (Baird, 2001; Remme *et al.*, 2001). A crucial step for malaria control is to make better and more informed decisions regarding anti-malarial drug use policy. This is dependent on the quality of the tools used for the detection and monitoring of resistance. Among four drug resistant assessment methods, molecular techniques are known with high accuracy and reproducibility for distinguishing the mutations.

Results obtained from molecular studies revealed that several point mutations in the *pfcrt* gene have been associated with resistance to chloroquine (Bloland *et al.*, 2000). According to the report of Johnson *et al.* (2004), point mutations in the trans-membrane protein *pfcrt* can not only determine whether *P. falciparum* is resistant to CQ but can also significantly influence parasite susceptibility to structurally unrelated anti-malarials. The results extend earlier observations on the role of *pfcrt* in contributing to parasite response to quinoline drugs (Sidhu *et al.*, 2002) and implicate this transporter as a key component underlying the complex cross resistance patterns observed in this organism (Johnson *et al.*, 2004).

The major molecular changes described to date include the replacement of a lysine (K) by a threonine (T) at position 76. This mutation is the most predictive of CQR, both *in vitro* and *in vivo*, suggesting that the characterization of this codon could be used as an epidemiological tool for large-scale studies of CQR in the field (Djimde *et al.*, 2001; Sidhu *et al.*, 2002).

The current methods for detection of these specific point mutations are laborious and time-consuming (Cheesman *et al.*, 2003). Real-time PCR does not require post PCR manipulations and the assay may be facilitated by high-throughput robotics. This closed-tube method also reduces contamination with PCR products that may interfere with subsequent assays (de Monbrison *et al.*, 2003).

In the present study, point mutations (*pfcrt* K76T, *pfcrt* SVIET and SVMNT as well as mix mutations) have been successfully detected in 28 blood samples using real-time PCR method. The SVMNT pattern detected in Chabahar is the same as the most prevalent resistant haplotype in India (Vathsala *et al.*, 2004). The CVIET is the same as the South East Asia (Pickard *et al.*, 2003). Three samples showed the deletion in amino acid located at position 75, Asparagine. Mixed infections (wild-type and mutant-type alleles) were detected among 11% of the major *pfcrt* alleles of which five samples (17.8%) showed two peaks simultaneously. It was probably consequence of mix-clone infections, contained two genotypes.

As the sensor probes are perfectly matched to the mutant-type allele, the melting temperature for mutant type alleles is higher than the melting temperature of wild type alleles.

In general, the sequencing results confirmed all the results obtained from real time PCR. In addition, real time PCR is considered as a sophisticated technique that can distinguish the mutations, reliably with acceptable speed, high accuracy, sensitivity and reproducibility. It is suggested to use real time PCR in association with *in vivo* test to provide fast and reliable complete early warning system for the detection and monitoring of CQ resistance in malarial areas.

REFERENCES

Babiker, H.A., S.J. Pringle, A. Abdel-Muhsin, M. Mackinnon, P. Hunt and D. Walliker, 2001. High-level chloroquine resistance in Sudanese isolates of *Plasmodium falciparum* is associated with mutations in the chloroquine resistance transporter gene *pfcrt* and the multidrug resistance gene *pfmdr*1. J. Infect. Dis., 183: 1535-1538.

Baird, J.K., 2001. Resurgent malaria at the millennium: Control strategies in crisis. Drugs, 59: 719-743.

Basco, L.K. and P. Ringwald, 2001. Analysis of the key *pfcrt* point mutation and *in vitro* and *in vivo* response to chloroquine in Yaounde. Cameroon. J. Infect. Dis., 183: 1828-1831.

Bloland, P.B., M. Euling and S. Mook, 2000. Combination therapy for malaria in Africa: Hype or hope? Bull. WHO., 78: 1378-1388.

Cheesman, S.J., J.C. de Roode, A.F. Read and R. Carter 2003. Real-time quantitative PCR for analysis of genetically mixed infections of malaria parasites: Technique validation and applications. Mol. Biochem. Parasitol., 131: 83-91.

de Monbrison, F., D. Raynauda, C. Latour-Fondanaichea, A. Staal and S. Favre *et al.*, 2003. Real-time PCR for chloroquine sensitivity assay and for *pfmdr1-pfcrt* single nucleotide polymorphisms in *Plasmodium falciparum*. J. Microbiol. Meth., 54: 391-401.

Djimde, A., O.K. Doumbo, J.F. Cortese, K. Kayentao and S. Doumbo *et al.*, 2001. A molecular marker for chloroquine-resistant *Falciparum* malaria. N. Eng. J. Med., 344: 257-263.

Dorsey, G., M.R. Kamya and P.I. Sigh, 2001. Polymorphism in the *Plasmodium falcipaium pfcrt* and *pfmdr*1 genes and clinical response to chloroquine Kampala, Uganda. J. Infect. Dis., 183: 1417-1420.

Edrissian, G.H. and S. Shahabi, 1985. Preliminary study of the response of *Plasmodium falciprum* to choroquine in Sistan and Baluchestan province of Iran. Trans. R. Soc. Trop. Med. Hyg., 79: 563-564.

Johnson, D.J., D.A. Fidock, M. Mungthin, V. Lakshmanan, A.B.S. Sidhu *et al*., 2004. Evidence for a central role for *pfcrt* in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents. Mol. Cell, 15: 867-877.

Lim, P., S. Chy, F. Ariey, S. Incardona and P. Chim *et al*., 2003. *pfcrt* polymorphism and chloroquine resistance in *Plasmodium falciparum* strains isolated in Cambodia. Antimicrob. Agents Chemother., 47: 87-94.

Mayor, A.G., X. Gomez-Olive, J.J. Aponte, S. Casimiro and S. Mabunda *et al*., 2001. Prevalence of the K76T mutation in the putative *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene and its relation to chloroquine resistance in Mozambique. J. Infect. Dis., 183: 1413-1416.

Pickard, A.L., C. Wongsrichanalai, A. Purfield, D. Kamwendo and K. Emery *et al*., 2003. Resistance to antimalarias in Southeast Asia and genetic Polymorphisms in *pfmdr*1. Antimicrob. Agents Chemother., 47: 2418-2423.

Pillai, D.R., A.C. Labbe, V. Vanisaveth, B. Hongvangthong and S. Pomphida *et al*., 2001. *Plasmodium falciparum* malaria in Laos: Chloroquine treatment outcome and predictive value of molecular markers. J. Infect. Dis., 183: 789-795.

Remme, J.H., F. Binka and D. Nabarro, 2001. Toward a framework and indications for monitoring Roll Back Malaria. Am. J. Trop. Med. Hyg., 64: 76-84.

Sangster, N., P. Batterham, H.D. Chapman, M. Duraisingh and L. LeJamber *et al*., 2002. Resistance to antiparasitic drugs; the role of molecular diagnosis. Int. J. Parasitol., 32: 637-653.

Sidhu, A.B., D. Verdier-Pinard and D.A. Fidock, 2002. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. Science, 298: 210-213.

Ursing, J., S. Zakeri, J.P. Gill and A. Bjorkman, 2006. Quinoline resistance associated polymorphisms in the *pfcrt*, *pfmdr*1 and *pfmrp* genes of *Plasmodium falciparum* in Iran. Acta Trop., 97: 352-356.

Vathsala, P.G., A. Pramanik, S. Dhanasekaran, C.U. Devi and C.R. Pillai *et al*., 2004. Widespread occurrence of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene haplotype SVMNT in *P. falciparum* malaria in India. Am. J. Trop. Med. Hyg., 70: 256-259.

Vieira, P.P., M. das Gracas Alecrim, L.H. da Silva, I. Gonzalez-Jimenez and M.G. Zalis, 2001. Analysis of the *pfcrt* K76T mutation in *Plasmodium falciparum* isolates from the Amazon region of Brazil. J. Infect. Dis., 183: 1832-1833.

Wongsrichanada, C., A.L. Pickard, W.H. Wernsdorfer and S.R. Meshnick, 2002. Epidemidogy of drug-resistant malaria. Lancet Infect. Dis., 2: 209-218.

Zakeri, S., S.T. Najafabadi, A. Zare and N.D. Djadid, 2002. Detection of malaria parasites by nested PCR in south-eastern Iran: Evidence of highly mixed infections in Chabahar district. Malaria J., 1: 2.