Genetic diversity at the C-terminal domain of knob-associated histidine-rich protein (KAHRP) of *Plasmodium falciparum* isolates from Burundi, Eastern Africa

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ABSTRACT

The knob-associated histidine-rich protein (KAHRP) is an exported parasite protein and the major component of infected erythrocytes by *Plasmodium falciparum*. *P. falciparum* histidine-rich protein-1 (PfHRP-1) is docked by KAHRP, which this interaction plays a significant role in cytoadherence of the malaria protozoan to erythrocytes and pathogenicity. The most polymorphic region of the PfHRP-1 is the C-terminal of decapeptide repeat domain (region III). The main objective of this study was to explore the genetic diversity at the region III of KAHRP in *P. falciparum* isolates from Burundi. In the present study, the nested PCR was performed for the amplification of the coding gene (*kahrp* gene) for *region* III in 35 *P. falciparum* isolates from Burundi. The nested PCR products of seven randomly selected isolates were purified and then sequenced. As the result, three allelic forms (340 bp, 370 bp, and 400 bp) were seen at the C-terminal domain of *kahrp* gene. The existence of multiple alleles of the *kahrp* gene revealed the presence of different *P. falciparum* strains in Burundi. It is suggested that the results could be useful in designing and the improvement of targeted therapy agents for *falciparum* malaria.

1. Introduction

Malaria is one of the most common infectious diseases worldwide. In 2015, most of the malaria cases (90%) and death for malaria (92%) were reported by world health organization (WHO). In addition, 5,159,706 malaria cases were reported from Burundi with 3799 deaths, which all caused by *P. falciparum* (http://apps.who.int/iris/bitstream/10665/252038/1/9789241511711-eng.pdf).

Among the *Plasmodium* species, which infected human population, *P. falciparum* is the main agent of severe malaria [1–3]. Cerebral malaria (CM) is main agent of mortality in *P. falciparum*-infected patients [1,3,4]. In these patients, the *P. falciparum*-infected and uninfected erythrocytes or red blood cells (RBCs) were accumulated in brain capillaries. The adherence of *P. falciparum*-infected RBCs to the vascular endothelial cells (cytoadherence) was carried out via cup-shaped nano-scale protrusions known as "knobs" at the surface of the parasitized RBCs. The knob-associated histidine-rich protein (KAHRP), also known as *P. falciparum* histidine-rich protein-1 (PfHRP-1), is a major component in the formation of knobs [5–9]. This 85-105- kDa protein is made by immature stages of the asexual life cycle of *P. falciparum*, and then transferred to the surface of RBCs [10,11].

The PfHRP-1 consists of three domains: the N-terminal histidine-rich (region I), the central lysine-rich (region II), and the C-terminal decapeptide repeat domains (region III) [12–14]. The C-terminal decapeptide repeat domain is the most polymorphic region of PfHRP-1 [13,15,16]. The parasitized RBCs are unable to adhere to endothelial cells without KAHRP [17]. In addition, it was showed that the knobless *P. falciparum* produced in vitro lost their virulence [6,11]. As the PfHRP-1 is one of the molecular therapy targets, the reagents that inhibit the formation of knobs will be effective in the treatment of severe

malaria [18]. Although the polymorphism of PfHRP-1 investigated in several malaria endemic areas [14–16,19,20], the present research is the first study which was conducted in Burundi, Eastern Africa. The aim of the present study was to explore the genetic diversity of region III of KAHRP in *P. falciparum* isolates from Burundi and compare the distribution frequency of PfHRP-1 alleles in the country.

2. Materials and methods

2.1. Sampling and DNA extraction

In this study, the finger prick blood samples were collected from 35 *P. falciparum*-infected patients attending to the malaria local clinics in Burundi between June 2014 and August 2015. Informed consent was taken from all the patients and or the parents before blood sampling. This study was approved by the Ethical Review Committee of Research at XXXXX University of Medical Sciences (IR-MAZ-EC- 95-2024), Iran, and Burundi.

All 35 *P. falciparum* isolates were diagnosed by direct microscopic examination of Giemsa-stained thick and thin blood smears. Then, one positive stained slide was chosen from each patient for DNA extraction. Genomic DNA was extracted from stained blood smears using Phenol: Chloroform: Isoamyl Alcohol (PCI) (Pasture institute of Iran, Tehran, Iran). To DNA extraction, the samples were wiped from each slide and transferred to microtubes. The smear rubbings were added to 200 μL lysis buffer containing 50 mM Tris-HCl, pH 7.5; 1 mM EDTA; 1% Tween 20; and 12 μL proteinase K (20 mg/mL). The microtubes were incubated at 56 °C for 2 h, and then, 200 μL of PCI was added. The mixture being shaken quickly, the tubes were spin at RPM 14,000 X for 10 min. Then, the supernatant was precipitated with 400 μL of absolute ethanol. Finally, 50 μL sterile distilled water was added to precipitated DNA and stored at 4 °C until it could be tested for *P. falciparum* DNA [21].

2.2. Nested PCR assay and DNA sequencing

A fragment of the *kahrp* gene was initially amplified by polymerase chain reaction (PCR) using Hp-F (5′-AATAATGGAAACGGATCCGGT GAC-3′) and Hp-R (5′−CCCTAACCACAGCATCCTG-3′) primers [19]. The amplification conditions for the first round of PCR were as following conditions: denaturation (95 °C, 5 min), 40x [(denaturation (94 °C, 1 min), (annealing 52 °C, 1 min), extension (72 °C, 1 min)], final extension (72 °C, 5 min). The primary PCR product was used as a template in the nested PCR to amplify the 402 bp region containing the C-terminal domain of the *kahrp* gene using Hn-F (5′-GAAACAAAAAA CACCGCTG-3′) and Hn-R (5′-GTACTGCATTAGCTCCTGTAGTTG-3′) primers [19].

In the second round, all conditions were the same as those for the first round, except the changes as 35 cycles and annealing temperature 57.5 °C. The nested PCR products were ran in the electrophoresis using 2.5% agarose gel containing SYBR green and visualized by ultraviolet (UV) transilluminator. The fragment size of products was determined using the DNA molecular weight marker.

In addition, seven nested PCR products were randomly purified and sequenced for three allelic types (Applied Biosystems 3730 DNA Analyzer, Bioneer Corporation, South Korea). The sequencing of the purified products was performed in both forward and reverse directions. The nucleotide sequence data of region III of *kahrp* gene from seven *P. falciparum* isolates were annotated in the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) database, which the assigned accession numbers are KX453289- KX453295.

2.3. Amino acid sequencing and phylogenetic analyses

The amino acid sequences of the seven *P. falciparum* isolates were observed and edited using Chromas version 2.33 (http://www.technelysium.com.au/chromas.html) (Technolysium Ltd.). Also,

Multalin [22] and ClustalW (http://www.genome.jp/tools/clustalw/) web tools were used to align kahrp nucleotide and deduced amino acid sequences among themselves as well as the accessible sequences of a number of *P. falciparum* isolates and reference strain, including NF7 (Ghana), FCR3 (Gambia), FCC1/HN (China), CO1 (Colombia), TZ5, TZ13 and TZ20 (Tanzania), HB5 and HB6 (Honduras), RJ181, Pf3-92 and Pf29-92 (India) and SB-1 to SB-18 and H-1 to H-10 (Iran). The BLAST (http://www.ncbi.nlm.nih.gov/blast) searches were performed in the GenBank database to determine the identity of reported *P. falciparum* isolates in this study with the kahrp gene sequences deposited in the GenBank. Phylogenetic analysis was performed with the construction of gene tree using the neighbor-joining (NJ) method in molecular evolutionary genetics analysis (MEGA) version 4.0 software [23]. The bootstrap method with 1000 replicates was used to assess the reliability of the gene tree.

2.4. Statistical analyses

Statistical analysis was conducted using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). In this analysis, a chi-square test with the significance limit of 0.05 was used to compare the frequency distribution of KAHRP alleles in Burundi.

3. Results

According to the nested PCR results, the isolates were identified into three allelic forms including, type I (340 bp), type II (370 bp), and type III (400 bp) (Fig. 1). The high molecular weight allele (400 bp) was predominant (23/35, 65.71%) with statistically significant difference ($P < 0.05$). The intermediate (370 bp) and low (340 bp) molecular weight alleles were observed in ten (28.57%) and two (5.72%) isolates, respectively. In addition, no isolate indicated multiple alleles (Table 1).

To more investigate and analyze the polymorphism, the nested PCR products of seven randomly selected isolates (four, two and one isolates with 400, 370 and 340 bp alleles, respectively) were purified and then sequenced. In this study, the nucleotide sequences of the C-terminal domain of the *kahrp* gene were identical unless one point mutation, as well as 30 and 60 deleted nucleotides (Fig. 2). The occurred point mutation at nucleotide position 2384 (T/A) of BR-4 and BR-5 isolates was synonymous, which it consequently had not been led to any amino acid change (Figs. 2 and 3). In comparison to the NF7 strain (as the reference sequence of the *kahrp* gene from *P. falciparum*), 30 and 60 nucleotides were deleted in two (BR-4 and BR-5) and one (BR-2) isolates, respectively (Fig. 2). The comparison of the amino acid sequences of seven isolates showed that either one (EATKEASTSK) or two (EAT-KEASTSKEATKEASTSK) of the incomplete decapeptides were eliminated in two (28.57%) and one (14.29%) isolates, respectively. No deletion was occurred in the remaining four (57.14%) isolates (Fig. 3). Thus, the number of decapeptide repeating units of the C-terminal domain (region III) of the *kahrp* gene was exchanged from three to five units in the 35 *P. falciparum* isolates from Burundi (residues 548-597 in Fig. 3). These repeating units contain two subunits, including one tetrapeptide and hexapeptide. The nested PCR results were confirmed using sequencing findings.

The phylogenetic tree of *kahrp* gene from *P. falciparum* was displayed in Fig. 4. The DNA sequencing and phylogenetic analysis approved the presence of three allelic forms (340 bp, 370 bp, and 400 bp) at the C-terminal domain of kahrp gene. The 1, 3, 6, and 7 isolates (KX453289, KX453291, KX453294 and KX453295) showed 100% identity to H-3 (HM776847), H-4 (HM776848), and SB-8 (HM776842) Iranian isolates and the NF7-Ghana reference strain (Y00060). The 4 and 5 isolates (KX453292 and KX453293) showed 100% identity to H-1 (HM776845), H-5 (HM776849), H-7 (HM776851), SB-1 (HM776835), and SB-9 (HM776843) Iranian isolates. The 2 isolate (KX453290) exhibited 100% identity to isolate TZ20 (AF124363) from Tanzania.
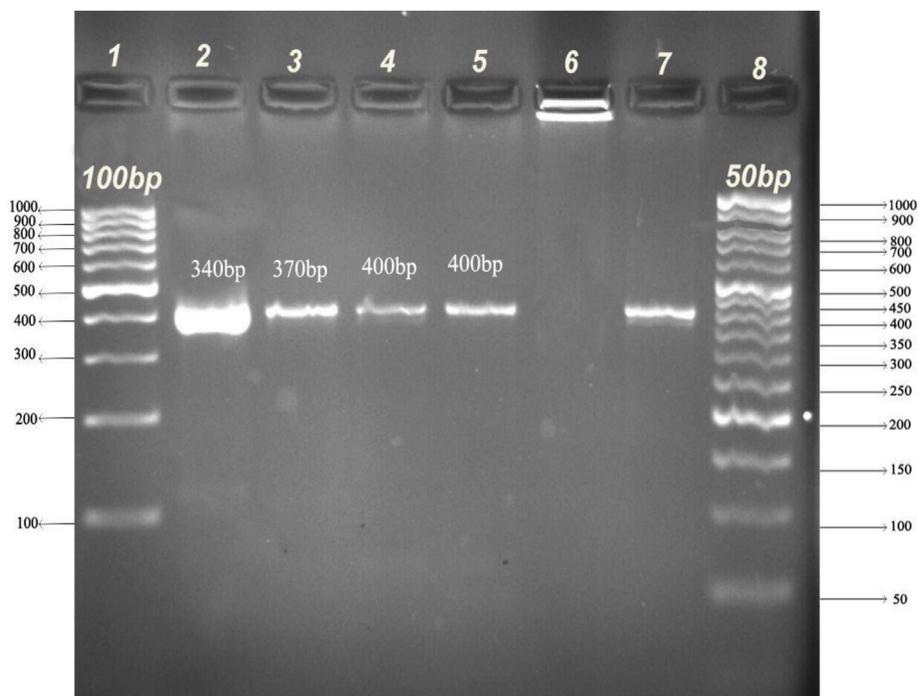
Fig. 1. Electrophoresis of nested PCR products of the region III of the *kahrp* gene from *P. falciparum* on 2.5% agarose gel. Lanes 1 and 8: DNA ladders (100 and 50 bps); lanes 2-5: patient samples; lane 6: negative control (dH₂0); lane 7: positive control (Iranian *P. falciparum* isolate, accession number HM776846).

Table 1
Allelic types of the C-terminal domain of *Plasmodium falciparum* kahrp gene based on the nested PCR and sequencing results in the isolates from Burundi.

Allelic forms	Amplicon size	Frequency (%)	Allele
I	340 bp	2 (5.72)	TKGA + 3 decapeptides + TKGA
II	370 bp	10 (28.57)	TKGA + 4 decapeptides + TKGA
III	400 bp	23 (65.71)	TKGA + 5 decapeptides + TKGA
Total		35 (100)	

4. Discussion

The KAHRP plays a significant role in the pathogenicity of *P. falciparum* infections [3] and is one of the potential targets for treatment of malaria [18]. Therefore, the investigation of polymorphism of KAHRP is significant in the designing and the development of a molecular therapy method.

Based on the nested PCR results, three allelic forms (340 bp, 370 bp, and 400 bp) were detected at the C-terminal domain of kahrp gene. Like the previous study performed in Iran [20], the type III (400 bp) allele was predominant (23/35, 65.71%) in the 35 Burundian *P. falciparum*

isolates and their molecular weight was the same size as NF7-Ghana, Pf3-92 from India, and 26 Iranian *P. falciparum* isolates [8,19,20]. The isolates with 370 bp allele were identical to the HB3-Honduras, Indian RJ181, and 20 isolates from Iran. The phenotype of the low molecular weight alleles (340 bp) was similar to the FCR3-Gambia, Pf 29-92 from Indian, and the three isolates of Iran [19,20].

The alignment of amino acid sequences demonstrated that the decapeptide repeating units of the BR-1, BR-3, BR-6, and BR-7 isolates with 400 bp allele were entirely the same as those of the NF7-Ghana strain and the 15 Iranian *P. falciparum* isolates including, SB-2, -3, -5, -6, -8, -10, -12, -15, -16, -18 and H-2, -3, -4, -6, and -10. In the BR-4 and BR-5 isolates with 370 bp allele, these units were exactly similar to those of the CO1 (Colombia) and the 10 Iranian *P. falciparum* isolates (SB-1, -9, -13, -14, -17 and H-1, -5, -7, -8, and -9). The decapeptide repeating units of the BR-2 isolate with 340 bp allele were completely similar to the TZ20 (Tanzania), FCC1/HN (China), and the three isolates of Iran (SB-4, -7, and -11), as well as FCR3-Gambia strain (Fig. 3). Moreover, according to the BLAST homology searches and phylogenetic analysis results of this study and a previous study conducted in Iran [20], it is appear that the Iranian *P. falciparum* parasites originates from Africa. Thus, further epidemiological and molecular investigations will help us to clear the hypothesis.

The results of our study revealed that the region III of the *kahrp* gene was polymorphic, as it had been already showed [13,15,16,20]. The
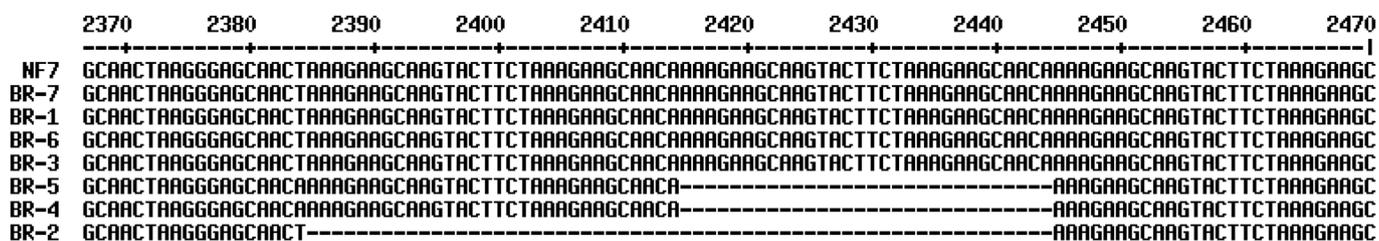
Fig. 2. Aligned nucleotide sequences comparison of the C-terminal domain of the *kahrp* gene of the seven *P. falciparum* isolates from Burundi with the NF7-Ghana strain (accession no. Y000060). NF7-Ghana is the only *P. falciparum* strain that its *kahrp* gene has been fully sequenced and is available. Dashes represent deleted nucleotides. BR: Burundi.
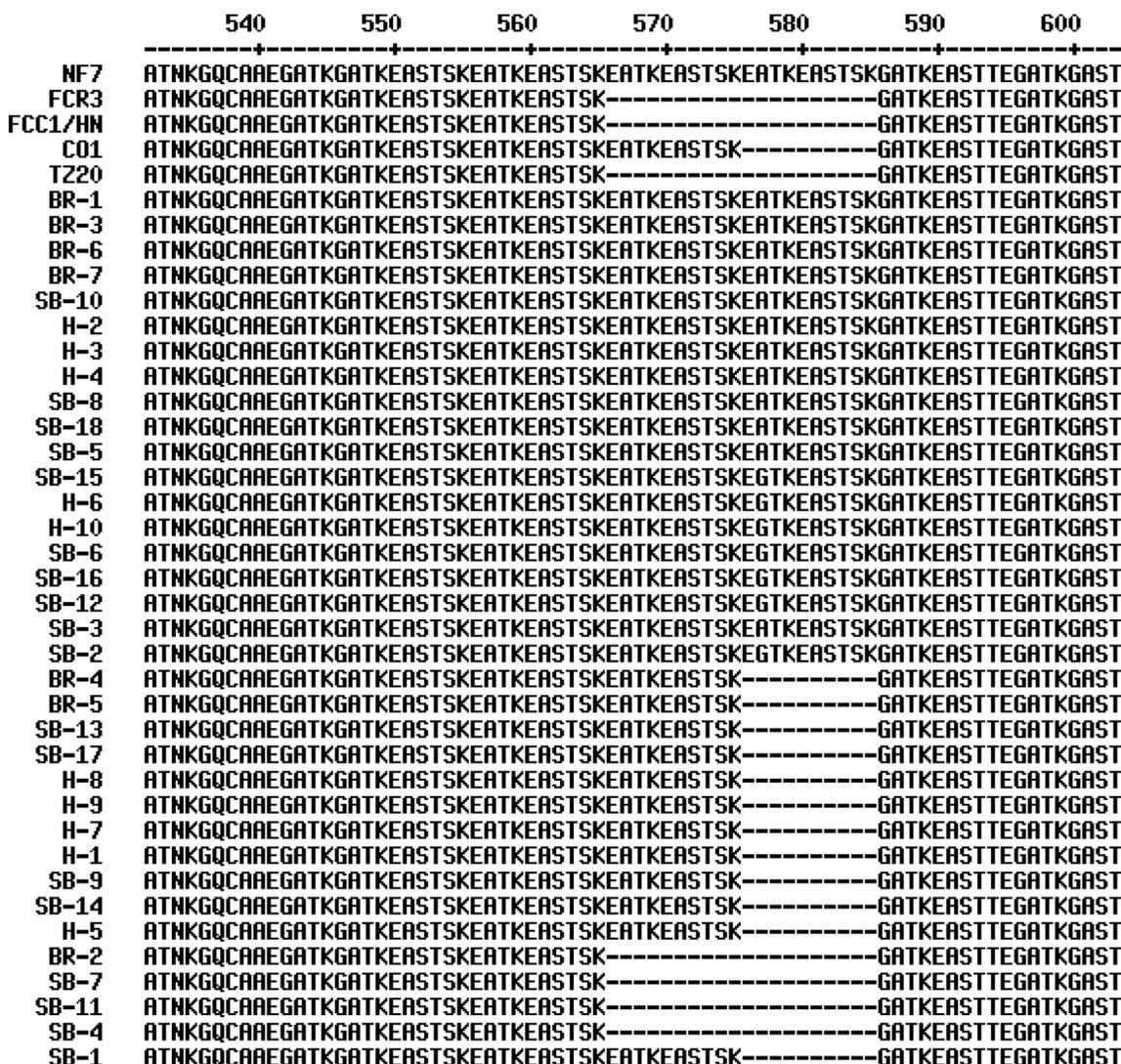
Fig. 3. Alignment of decapeptide repeating units of the C-terminal domain of the *kahrp* gene in the seven isolates (BR -1 to BR-7) of P. *falciparum* from **Burundi**. The amino acid sequences were compared with the available sequences of the NF7-Ghana and FCR3-Gambia strains, as well as FCC1/HN (China), CO1 (Colombia), TZ20 (Tanzania), and the 28 (SB-1 to SB-18 and H-1 to H-10) Iranian isolates (accession numbers P06719, P09346, AF275687, AF124364, AF124363, HQ853688-98, and HM776835-51, respectively). Dashes represent deleted nucleotides. BR: Burundi, SB: Sistan va Baluchestan province, H: Hormozgan province.

existence of multiple allelic forms of the *kahrp* gene identified the presence of different *P. falciparum* strains in Burundi. Since malaria is highly common in Burundi (http://apps.who.int/iris/bitstream/10665/252038/1/9789241511711-eng.pdf), the presence of this of infectious disease can be explained due to the higher diversity of the C-terminal domain of the *kahrp* genes in the *P. falciparum* isolates.

5. Conclusion

Since the presence of the polymorphism in the *P. falciparum* antigen candidates, currently there is no safe and potential vaccin against the infection [6,24]. Furthermore, the resistance of *P. falciparum* to antimalaria medications has been reported from almost all malaria endemic regions of the world (http://apps.who.int/iris/bitstream/10665/252038/1/9789241511711-eng.pdf). Therefore, the alternative control strategies such as molecular therapy should be used in the endemic areas. Since the KAHRP is the main ingredient of knobs [5,7], it appears to be an appropriate candidate for anti-adhesion therapy. The results of this study could be useful in the improvement of targeted therapy for *falciparum* malaria.

Conflicts of interest

The authors declare that they have no conflict of interests.

Ethical approval

This study was approved by the Ethical Review Committee of Research at Mazandaran University of Medical Sciences (IR-MAZ-EC-95-2024).

Consent

Our study was done on archived blood smears of malaria patients.
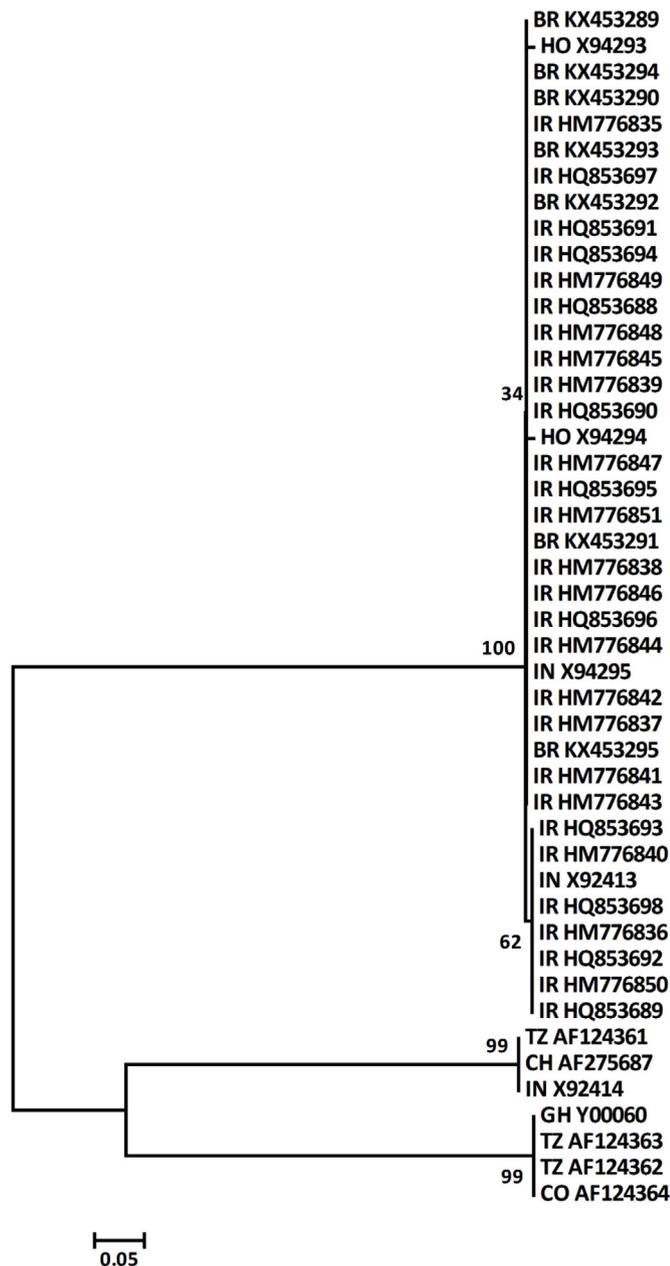
Fig. 4. Phylogenetic tree of the *kahrp* gene of *P. falciparum*. The tree was constructed using the neighbor-joining method (NJ) with Tamura 3-parameter genetic distance model in the molecular evolutionary genetics analysis (MEGA) version 4.0 software (Tamura et al., 2007). The numbers on and/or under the branches indicate bootstrap values according to 1000 replicates. Besides the kahrp gene sequences of seven *P. falciparum* clinical isolates this study (BR KX453289-KX453295), the kahrp gene sequences of a number of *P. falciparum* isolates and reference strain (NF7, FCC1/HN, CO1, TZ5, TZ13, TZ20, HB5, HB6, RJ181, Pf3-92, Pf29-92, SB-1 to SB-18 and H-1 to H-10) from several countries were applied with their accession numbers in phylogenetic analysis, including Ghana (GH), China (CH), Colombia (CO), Tanzania (TZ), Honduras (HO), India (IN) and Iran (IR).

Author contribution

Design: Ahmad Mardani, Mahdi Fakhar, Hajar Ziaei Hezarjaribi.
Data collection and sampling: Seyed Naser Emadi, Azadeh Rezaei-rad, Joseph Butore, Ndayikunda Claudete.
Data analysis: Mahdi Fakhar, Ahmad Mardani.
Writing the paper: Mahdi Fakhar, Ahmad Mardani.

Registration of research studies

We have done the study only on the blood smears not on human.

Guarantor

Ahmad Mardani, Mahdi Fakhar.

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