An unlabeled probe based real time PCR and modified semi nested PCR as molecular tools for analysis of chloroquine resistant in *Plasmodium vivax* isolates from Afghanistan

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Research

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Abstract

Background: Chloroquine (CQ) resistance *Plasmodium vivax* isolates have been reported from many endemic regions in the world. *P. vivax* has been reported to be about 95% of the whole malaria in Afghanistan and CQ is prescribing in the first-line treatment of *vivax* malaria. The *pvmdr-1* and *pvcrt-o* (K10 insertion) genes are the possible markers of CQ-resistance *P. vivax* isolates. There have been no studies done on the prevalence of molecular markers of CQ-resistance *P. vivax* in Afghanistan. In the present work, we aimed to evaluate the prevalence of mutations in the *pvmdr-1* and K10 insertion in the *pvcrt-o* genes of *P. vivax.*

Methods: *P. vivax* isolates were collected from Laghman, Baghlan and Khost provinces. For investigation of polymorphisms of desired regions in *pvmdr-1 and pvcrt-o* genes, sequencing was applied on the PCR products. We developed a new asymmetric qPCR and melting analysis assay based on unlabeled probe for scanning of K10 insertion in *pvcrt-o* gene.

Results: The analysis of sequencing data of the *pvmdr-1* gene showed wild type Y976 and K997 and mutant M958 and L1076 in 33 isolates from three provinces and submitted in GenBank (accession number MK419882-MK419914)

Of 36 samples that evaluated for K10 insertion in *pvcrt-o*, 2/18(11%), 0/10(0%) and 0/8(0%) isolates from Laghman, Baghlan and Khost province possessed K10 insertion, respectively, that confirmed by either sequencing and unlabeled probes and submitted in GenBank (accession number MK292011-MK292046).

Conclusion: The existence of 2 samples with K10 insertion and 33 samples with *pvmdr1* polymorphism indicating on CQ resistance *P. vivax* populations in Afghanistan. This can lead to spreading of resistant strains in the society. Furthermore, unlabeled probes are simple and inexpensive alternative tools for screening of *P. vivax* mutations.

Background

A total of 216 million estimated cases of malaria was reported from 91 countries during 2016. Afghanistan possesses 13% (138217) of malaria cases in eastern Mediterranean region and 95.6% (132237) of the cases were related to *Plasmodium vivax*[1]. *Plasmodium vivax*, because of its low mortality, is one of the neglected causative agents of malaria in the world, which has the widest geographical distribution among the other four types of human Plasmodium [2]. Most tropical areas like Middle East, Asia, and the Western Pacific, account for about 80-90% of vivax malaria outside of Africa [3]. Although many researchers believe that severe malaria (characterized by cerebral malaria, renal failure, circulatory collapse, severe anemia, hemoglobinuria, abnormal bleeding, ARDS, and jaundice) caused by *Plasmodium falciparum*, there are certain evidence showing that *Plasmodium vivax* can also cause severe malaria in humans [4]. The CQ-resistant *P. falciparum* was first reported in1959 while CQ-resistant *P. vivax* was first reported in 1989, when first detection of CQ-resistant *P. falciparum* was detected in Afghanistan [5-7]. The resistance of the *Plasmodium falciparum* to chloroquine was very

unpleasant, which took the lives of so many humans. Therefore, to avoid such a disaster in the case of *Plasmodium vivax*, extensive, rapid and profound research must be conducted [8]. Of major causes of failure to control and eradicate vivax malaria are the emergence of antimalarial drug resistant cases and its ability to go to the dormant state that relapses weeks to months after the initial infection [9].

The first cases of chloroquine-resistant *Plasmodium vivax* has been reported from Papua New Guinea and after that was observed in other parts of the world [5]. While reports of Chloroquine (CQ) resistant *vivax* infections are increasing, this drug is still the primary therapy against *Plasmodium vivax* malaria in many endemic countries[6]. Chloroquine has been widely used in the treatment of uncomplicated malaria in Afghanistan since the 1940s, and remains the most widely used antimalarial drug[10].

Although there are few studies have been done on the assessment of resistant vivax malaria, no work has been done for evaluation of CQ efficacy on vivax malaria in Afghanistan [11-13]. Many studies have been conducted to analyze the point mutations in the pvmdr1 gene on chromosome 5 and *pvcrt-o* gene on chromosome 1 and to evaluate their relation to the resistance of *Plasmodium vivax* to chloroquine [14]. Although so many researches have been conducted on these two genes throughout the world, there has been no research on the prevalence of these mutations in Afghanistan. There are several methods to analyze point mutations such as PCR-sequencing, ARMS-PCR, PCR-RFLP. One of the new techniques for the detection of polymorphisms of genes is asymmetric qPCR and using unlabeled probes [15]. In this study, for the first time we investigated the insertion of K10 in the *pvcrt-o* gene using a modified semi nested PCR-sequencing and asymmetric qPCR method. Successful and accurate detection of drug-resistant *P. vivax* parasites may help to malaria control, treatment and elimination strategies [16]. In this work we evaluated the mutations of CQ-resistant associated genes (*pvmdr1* and *pvcrt-o*) in *Plasmodium vivax* isolates from three endemic provinces of Afghanistan.

Methods

Locations, samples collection and DNA purification

In general,50,15, and 15 *P. vivax* microscopically confirmed cases were collected from the patients that were referred to malaria diagnostics centers in Laghman (34° North, 70° East), Baghlan (36° North and 68° East) and Khost (33° North and 70° East) provinces (Fig. 1) respectively, during 2017. For this propose finger stick blood samples were spotted on DNA Banking Cards (DBCs) (Kowsar Biotechnology Center, Tehran, Iran) and microscopic slides.

DNA extraction and *Plasmodium vivax* confirmation:

Disks (2 mm in diameter) were punched out from each DBCs and washed 3 times with DBCs purification buffer and twice with distilled water. The disks were air dried and used directly as template of PCR processing [17].A semi-nested multiplex PCR were performed on each sample for final confirmation of *P. vivax*[18].

Evaluation of *pvmdr1* mutations by nested-PCR:

Briefly in first round *Pvmdr-1* (OF) and *Pvmdr-1* (OR) primers, were applied for amplification of about 967 bp fragment of pvmdr1 gene of Plasmodium vivax. Three punched discs were used for each PCR reaction of the first round. One microliter of all products of the first round diluted into 500 µl of sterile water. In the second round the 1 µl of *Pvmdr-1* (NF) and *Pvmdr-1* (NR) primers and two microliter of diluted product of first roundwere added into 25 microliter of "2X Taq Master Mix Red" (Amplicon inc., containing 150 mMTris-Cl PH 8.5, 40 mM (nh4)2So4, 3 mM Mgcl2, 0.2% tween 20, 0.4 mMdntPs, 0.05 U/µl Taq DNA polymerase, inert red dye and stabilizer), to reaching final concentration of 50µl. For the first round condition was done in 94°C, 5 min; 30 cycles of 94°C, 15 sec; 60°C, 30 sec; 72°C, 1 min; 72°C, 7 min, while the second round PCR was done under the following conditions: 94°C, 5 min; 30 cycles of 94°C, 15 sec; 57°C, 30 sec; 72°C, 1 min; 72°C, 7 min. The products of the second round (604 bp) were seen in 2% agarose gels (SYBR Safe stain; Invitrogen; Groningen, The Netherlands). The *Pvmdr-1* (NF) and PCR products were sent for sequencing by the ABI3730XL sequence analyzer (Macrogen, Korea) [8].

Table 1. Primers used for amplifications of *pvcrt-o* and *pvmdr-1* marker genes: OF (Outer forward), OR (outer reverse), NF (nested forward), NR (nested reverse).

Primers	Sequences 5' → 3'	Final Size (bp)	Annealing temp. (°C)
Pvmdr-1 (OF)	CGCCATTATAGCCCTGAGCA	604	60
Pvmdr-1 (OR)	TCTCACGTCGATGAGGGACT		
Pvmdr-1 (NF)	GGATAGTCATGCCCCAGGATTG		57
Pvmdr-1 (NR)	CATCAACTTCCCGGCGTAGC		
Pvcrt-o F	AAGAGCCGTCTAGCCATCC	296	52
Pvcrt-o R	AGTTTCCCTCTACACCCG		
Pvcrt-o (Rseq)	GGGGACGTCCTCTTGTATTT		55

Evaluation of *pvcrt-o* K10 insertion by semi-nested PCR:

Briefly in first round *pvcrt-o* (OF) and *pvcrt-o* (OR) primers, were applied for amplification of about 1186 bp fragment of *pvcrt-o* gene of *Plasmodium vivax*. Three punched discs were used for each PCR reaction of the first round. One microliter of all products of the first round diluted into 500 µl of sterile water. In the second round the 1 µl of *Pvcrt-o*(OF) and *Pvcrt-o* (Rseq)primers and two microliter of diluted product of first round were added into 25 microliter of "2X Taq Master Mix Red" (Amplicon inc), to reaching final concentration of 50 µl. For the first round condition was done in 95°C, 5 min; 30 cycles of 94°C, 15 sec; 52°C, 30 sec; 72°C, 90sec; 72°C, 7 min, while the second round PCR was done under the following

conditions: 94°C, 5 min; 30 cycles of 94°C, 15 sec; 55°C, 30 sec; 72°C, 30 sec; 72°C, 7 min. The products of the second round (296 bp) were seen in 2% agarose gels (SYBR Safe stain; Invitrogen; Groningen, The Netherlands). The *Pvcrt-o*(OF) and PCR products were sent for sequencing by the ABI3730XL sequence analyzer (Macrogen, Korea) [8, 19].

Development of asymmetric real time PCR and melt-curve analysis

Primer and probe design

We imported genomic sequence of the *Plasmodium vivax* (accession number: EU33972) into CLC Main Workbench 5 (CLC bio, Aarhus, Denmark) Software. We selected *Pvcrto*-OF primer as forward primer [8] and designed a reverse primer for *pvcrt-o* gene. We also designed a probe to contain the insertion of interest. The probe blocking improved by amino-modified C6 to prevent extension during PCR amplification. The primers and probe sequences and their position in the *pvcrt-o* gene are presented in Table 2 and Figure 2 respectively.

Table 2: High-resolution melting assay primer and probe sequences used for detection K10 insertion in *pvcrt-o* gene.

Name	Primer/ probe	Sequence 5' → 3'	TM	Products size	
				Wild	Mutant
Pvcrto-OF	Forward primer	GCTACCCCTAACGCACAATG	60	80	83
Afg.HRM. R	Reverse primer	CCGGTAACGTTCATCGG			
Afg.U.P	Unlabeled Probe	CTGAAAAAGAAGAAGAAGAAGGG- block			

Amplification conditions

The qPCR-HRM assay was performed using an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Inc.). The PCR was set up in a final volume of 20 µl containing 0.1 µM of Pvcrt0-OF (forward primer), 0.5 µM Afg.HRM. R (the reverse primer and the excess primer) and 0.5 µM Afg.U.P probe, 2 µl of diluted product of first round from semi nested PCR and 4 µl of 5X Hot Firepol® EvaGreen® HRM Mix (Solis BioDyne, Tartu, Estonia) in qPCR 8-strip tubes (Gunster Biotech, Taiwan).

The thermal program included an initial denaturation at 95 °C for 12 minutes, followed by 40 cycles of amplification consisting of 95 °C for 15 sec (denaturation step), 60 °C for 20 sec (primer annealing), and 72 °C for 20 sec (elongation step). The amplicons were then subject to a melt program for dissociate the double stranded DNA at 95 °C for 15 seconds, gradual temperature for HRM increase from 55 °C for 1 minute until 95 °C for 15 sec at a thermal transition rate of 0.3%. Ultimately, obtained melting curve

profiles were analyzed by making a use of HRM software for Windows® version 3.0.1. (Applied Biosystems).

Results

Verification of *Plasmodium vivax* samples

All microscopically confirmed *P. vivax* isolated were also verified by semi-nested multiplex PCR and then applied for evaluation of mutations in *pvmdr1* and *pvcrt-o* genes.

Evaluation *pvmdr1* mutations by nested-PCR:

The *pvmdr-1* gene was successfully amplified and sequenced in 92% (33/36) of the *P. vivax* isolates. In spite of resequencing, 3 (8%) of the samples had not acceptable sequencing results.

The analysis of sequencing data of the *pvmdr-1* gene (Fig. 3) showed wild type Y976 and K997 and mutant M958 and L1076 in all isolates from three provinces and submitted in GenBank, MK419882-MK419914 (Table 3).

Table 3 Distribution of pvmdr1 mutations among *P. vivax* isolates from Laghman, Baghlan and Khost.

	No. isolates (%) from the following provinces			Mutations (SNP)
Frequency (%)	Khost	Baghlan	Laghman	
33 (100)	7(100)	9 (100)	17(100)	T958M (ACG/ACG)
0 (0)	0(0)	0(0)	0(0)	Y976F (TAC/TTC)
0 (0)	0(0)	0(0)	0(0)	K997R (AAG/AGG)
33 (100)	7(100)	9 (100)	17(100)	F1076L (TTT/CTT)
33 (100)	7	9	17	total

Evaluation of K10 insertion in pvcrt-o gene by unlabeled probe and semi-nested PCR

The *pvcrt-o* gene was successfully amplified and sequenced in 100% (36/36) of the *P. vivax* isolates.

Of 36 samples that evaluated for K10 insertion in *pvcrt-o*, 2/18(11%), 0/10(0%) and 0/8(0%) isolates from Laghman, Baghlan and Khost province possessed K10 insertion, respectively, that confirmed by either sequencing and unlabeled probes and submitted in GenBank, MK292011-MK292046 (Table 4).

Table 4: Distribution of *pvcrt-o* K10 insertion among *P. vivax* isolates from Laghman, Baghlan and Khost.

Frequency (%)	No. isolates (%) from the following provinces			polymorphism
	Khost	Baghlan	Laghman	
2 (5.55)	0(0)	0 (0)	2(11.11)	K10 Insert
36 (100)	8	10	18	total

High resolution melting assay primer and Unlabeled probe

Simultaneous amplification and screening of mutant and wild types of *pvcrt-o* gene was performed by qPCR-HRM and unlabeled probe. Unfortunately, PCR product melting transitions were not able to independently identify the genotype. The whole melting profile, showing the melting region of both the unlabeled probe and the PCR products, is shown in Fig.1. Two peaks were apparent. If K10 insertion existed, the probe melted at 55.5–64 °C, whereas the PCR product melted at 78.5–83 °C (Fig. 4).

Discussion

Malaria is endemic in the most parts of Afghanistan, and more than 95% of reported cases are *Plasmodium vivax*, for which chloroquine is used as first-line therapy [1]. So, researches on the prevalence of molecular mutations in the genes that are responsible for chloroquine resistance, such as *pvcrt-o* and *pvmdr1*, is necessary. Evaluation of these mutations has not been conducted in Afghanistan and this work is the first report of its type.

Y976F mutation in *pvmdr1* gene as well as the K10 insertion in the *pvcrt-o* gene are considered most responsible for *Plasmodium vivax* resistance to chloroquine [20, 21]. The Y976F mutation is prevalent in high-grade chloroquine-resistant foci (Thailand and Myanmar) compared with low-grade regions (Republic of Korea) [22]. In a study from Thailand, the Y976F and F1076L mutations reported 23% and 53%, respectively [23]. As in the present study, the F1076L mutation was reported in 100% isolates of Republic of Korea, Madagascar and Mauritania [22, 24, 25]. Although Y976F and K997R mutations were not observed in any of the isolates of *Plasmodium vivax* in Afghanistan. The presence of two mutations F1076L and T958M in all isolates suggests a change in parasitic genotype, which may lead to a modest positive change to reach the chloroquine resistant phenotype.

The insertion of AAG (codon of Lysine) was present in 56.0% and 46.2% of isolates from Thailand and Myanmar, respectively. The presence of K10 insertion in two isolates from Laghman province, which has shown an ascending trend in cases of *Plasmodium vivax* in the last 10 years, is an alarm for the public health authorities in Afghanistan.

In several studies conducted on the *pvcrt-o* gene, appropriate internal primers that can replicate K10 insertion were not used, and these primers replicated on the points further, such mutations did not exist at all [8, 23, 25]. In addition, due to the low copy number of this gene in the *Plasmodium vivax*, use of one

pair of primers for amplification of the K10 insertion is not suitable for samples with low parasite loading [19]. In this study, we used a modified semi-nested PCR method. In this method we used two primers of Lu et al (2012) study for the amplification of *pvcrt-o* gene [19]. In the second round, we used the forward primer of first round and the *Rseq* primer of Glossa et al (2015) study [8]. Finally, we saw the sharp bands suitable for sequencing. Although the sequencing method is the most accurate method for SNP genotyping, but it is expensive, time consuming and in some circumstances impossible due to lack of infrastructures. On the other hand, the PCR-RFLP is cheaper and faster, but this method lacks sufficient enzymes for all mutations. Melt curve analysis and the use of the unlabeled probe in asymmetric qPCR is a new method used in various studies. The use of this method is much affordable as unlabeled probes are much cheaper than enzymes and can be easily transferred than enzymes. Instead, HRM analysis may be enough to detect mutations, but the use of an unlabeled probe signifies the accuracy of the work. The qPCR does not have post-PCR manipulation problems, seen in sequencing and PCR-RFLP. In the present study, due to financial problems, we could not investigate all the mutations by using the unlabeled probe method and we only examine the K10 insertion. Afghanistan is a country with other challenges, and this made us unable to investigate the number of copies of the genes, in-vitro study, and the clinical responses of *P. vivax* isolates. In fact, this work is a snapshot of chloroquine *p. vivax* resistance situation in Afghanistan. Further researches should also be conducted on a large number of samples throughout Afghanistan. We hope that the results of this research will be the basis for other researchers. To study the actual relationship between the mutations in the *pvmdr1* and *pvcrt-o* genes and the drug resistance we suggest, first, to study the effect of these mutations on the final form of proteins by molecular dynamic and protein modeling, and then the interactions between these malformed proteins and chloroquine in-silico environment. In fact, genomic, transcriptomic and proteomic should be used simultaneously.

Conclusions

Chloroquine resistant *Plasmodium vivax* is a neglected risk in regions with other challenges like Afghanistan. Lack of data impedes estimating and addressing the burden this risk. This study is an example of a study providing a baseline data that can contribute to the discussion about the neglected status of chloroquine resistant *Plasmodium vivax* in the region and subsequently in global scales. Indeed, further in-vitro and clinical observation studies must be done for better understanding of chloroquine resistant *Plasmodium vivax* status in Afghanistan. Despite existence of molecular equipment in Afghanistan, a gap feels in applying these methods in either health or academic sectors.

Abbreviations

P. vivax: Plasmodium vivax

P. falciparum: Plasmodium falciparum

Pvcrt-o: Plasmodium vivax chloroquine resistant transporter

Pvmdr-1: *Plasmodium vivax* multidrug resistant

HRM: High Resolution Melting

qPCR: Quantitative Polymerase Chain Reaction

DBCs: DNA Banking Cards

RFLP: Restriction fragment length polymorphisms

Declarations

Ethics approval and consent to participate

The study proposal was reviewed and approved by Medical Ethic Committee of Tarbiat Modares University, Tehran, Iran (IR.TMU.REC.1395.401). Informed consent was taken from all people participated in this study.

Consent for publication

Not applicable

Availability of data and materials

All datasets are included within the paper. Any question may be obtained from the corresponding author upon request.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SHM and NS managing the sample collection. SHM and RFA performed the bioinformatics analysis and experiments. AD corresponded the work. NS, RFA, JS and FG are the advisors. SHM, AD and NS drafted the manuscript. All authors read and approved the final manuscript.

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Figures

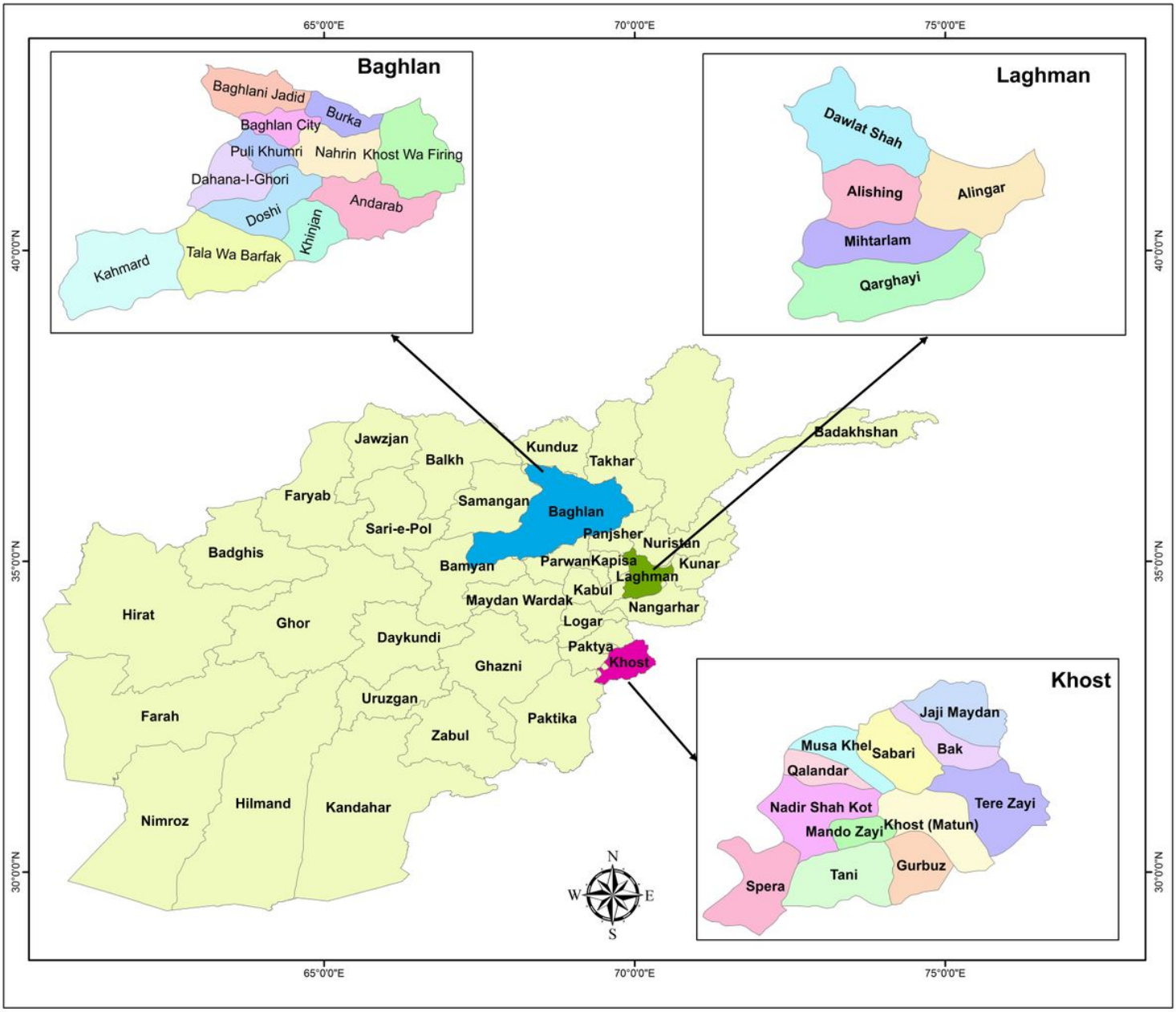

Figure 1

Geographical map of Laghman, Baghlan and Khost provinces in Afghanistan

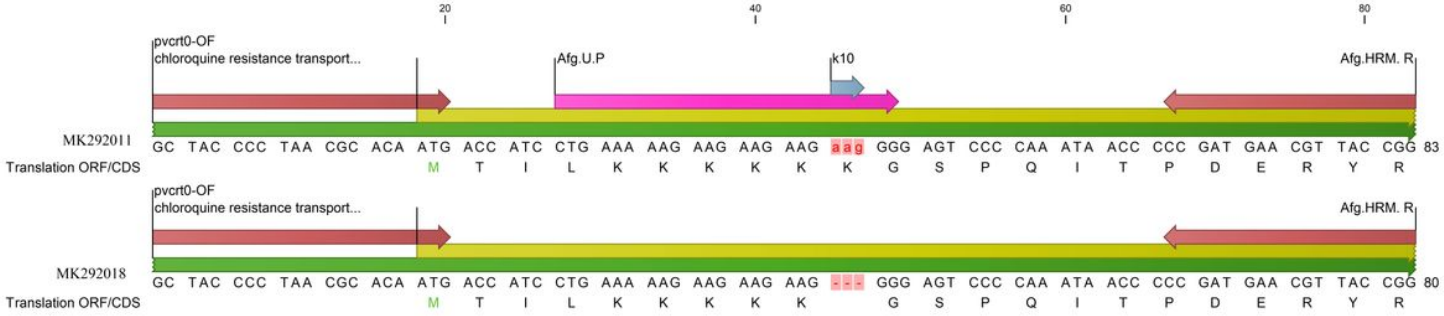

Figure 2

Schematic map of the pvcrt-o gene and applied primers and unlabeled probe in asymmetric qPCR: The position of primers (red flashes), unlabeled probe (purple arrow), K10 insertion (blue arrow), coding DNA sequence (yellow line) and mRNA (green line) illustrated by using of CLC bioinformatics software.

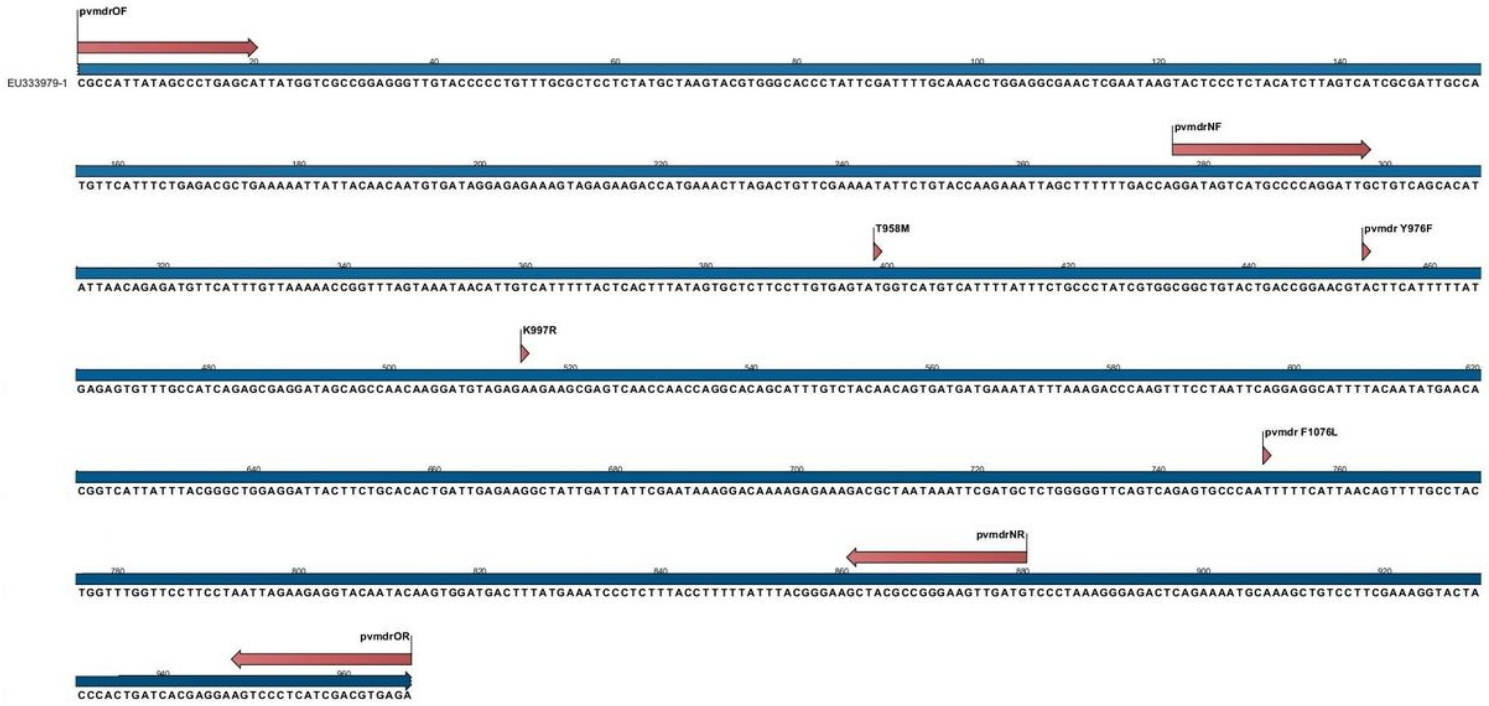

Figure 3

Schematic map of the pvmdr-1 gene: The position of primers (red flashes) and mutations (red arrows) illustrated by using of CLC bioinformatics software.

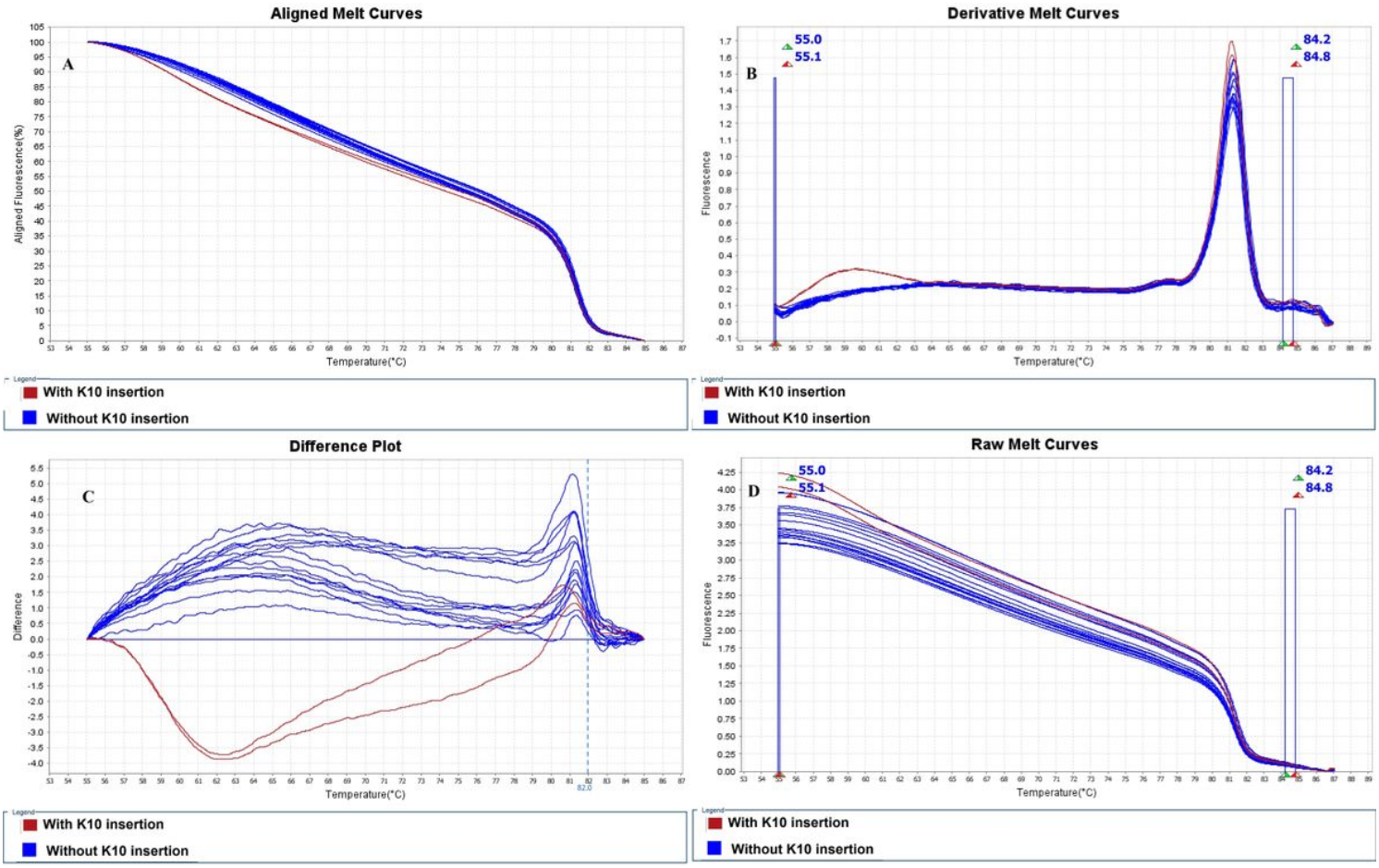

Figure 4

Comparison of melting temperature of pvcrt-o gene mutation by unlabeled probe: A: Aligned and normalized melt curves, B: Derivative melt curves, C: normalized difference plot and D: Raw Melt curve respectively. Separation of inserted (red) and non-inserted (blue) K10 variation.