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Research Article



DEVELOPMENT OF A TECHNIQUE FOR ANALYZING THE LENGTHS OF RESTRICTION FRAGMENTS TO ASSESS THE MEFLOROQUINE RESISTANCE OF *PLASMODIUM FALCIPARUM* BASED ON THE IDENTIFICATION OF SINGLE-NUCLEOTIDE POLYMORPHISMS (A958146T, A961013G, G961625T) OF THE *PFMDR1* GENE

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ABSTRACT

A method for identifying single nucleotide polymorphisms of the *PfMDR1* gene (A958146T, A961013G, G961625T) based on the analysis of the lengths of restriction fragments using polymerase chain reaction technology with specific primers is presented. To identify the polymorphism A958146T (amino acid substitution N86Y), it was proposed to amplify a fragment of parasitic deoxyribonucleic acid with a length of 417 bp, including the 86th codon of the *PfMDR1* gene, followed by treatment of the amplicon with *ApoI* endonuclease, the restriction site of which includes the sequence of the analyzed mutation. With the *Plasmodium falciparum* genotype unchanged, the isolated deoxyribonucleic acid site is divided into two fragments of size 239 and 179 bp. In the case of an altered genotype containing a single nucleotide missense mutation A > T, the original fragment 417 bp will be preserved. When designing the system to detect the amino acid substitution N1042D caused by the A961013G mutation, a pair of specific primers were selected that limit the 404 bp-long section of parasitic deoxyribonucleic acid. For restriction analysis, the most optimal was the use of *Asel* endonuclease, which in the case of an unchanged *Plasmodium falciparum* genotype divides the initial amplicon into 4 fragments (132, 116, 99 and 25 bp), and in the presence of A > G mutation — into 3 (248, 99, 25 bp). It was found that a fragment of the *PfMDR1* gene, including the G961625T mutation leading to the amino acid substitution D1246Y, contains 1 site corresponding to the restriction site of endonuclease *BglII*. Therefore, in the case of the wild genotype of *Plasmodium falciparum*, the initial fragment of deoxyribonucleic acid is cut into 2 short sections (300 and 269 bp). With the D1246 mutation, the replacement of the nucleotide G > T leads to the disappearance of the restriction site, so only one source fragment (509 bp) will be recorded on the electrophoregram. Based on the analysis of the data obtained, criteria for evaluating the drug resistance of *Plasmodium falciparum* have been developed. The decrease in sensitivity to mefloquine and its derivatives of pathogens of tropical malaria can be evidenced by positive results obtained using the developed methods for detecting haplotypes A > T (N86Y (band 417 bp)), A > G (N1042D (bands 248, 99 and 25 bp)), G > T (D1246Y (509 bp)). The developed methods can be used to analyze the spread of drug-resistant tropical malaria.

Keywords: tropical malaria; *Plasmodium falciparum*; nucleotide polymorphisms; restriction fragments; polymerase chain reaction; specific primers; amplification; amplicon; missense mutation.

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Научная статья

РАЗРАБОТКА МЕТОДИКИ АНАЛИЗА ДЛИН РЕСТРИКЦИОННЫХ ФРАГМЕНТОВ ДЛЯ ОЦЕНКИ МЕФЛОХИН-РЕЗИСТЕНТНОСТИ *PLASMODIUM FALCIPARUM* НА ОСНОВЕ ИДЕНТИФИКАЦИИ ОДНОНУКЛЕОТИДНЫХ ПОЛИМОРФИЗМОВ (A958146T, A961013G, G961625T) ГЕНА *PFMDR1*

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Резюме

Представлена методика идентификации единичных нуклеотидных полиморфизмов гена *PfMDR1* (A958146T, A961013G, G961625T) на основе анализа длин рестрикционных фрагментов с использованием технологии полимеразной цепной реакции со специфическими праймерами. Для идентификации полиморфизма A958146T (аминокислотная замена N86Y) предложено амплифицировать фрагмент паразитарной дезоксирибонуклеиновой кислоты длиной 417 bp, включающий 86-й кодон гена *PfMDR1*, с последующей обработкой ампликона эндонуклеазой AroI, сайт рестрикции которой включает последовательность анализируемой мутации. При неизменном генотипе *Plasmodium falciparum* выделенный участок дезоксирибонуклеиновой кислоты разделяется на 2 фрагмента размером 239 и 179 bp. В случае измененного генотипа, содержащего однонуклеотидную миссенс-мутацию A > T, сохранится исходный фрагмент 417 bp. При конструировании системы для выявления аминокислотной замены N1042D, обусловленной мутацией A961013G, была подобрана пара специфических праймеров, ограничивающих участок паразитарной дезоксирибонуклеиновой кислоты длиной 404 bp. Для проведения рестрикционного анализа наиболее оптимальным оказалось применение эндонуклеазы AseI, которая в случае неизменного генотипа *Plasmodium falciparum* делит исходный ампликон на 4 фрагмента (132, 116, 99 и 25 bp), а при наличии мутации A > G — на 3 (248, 99, 25 bp). Установлено, что фрагмент гена *PfMDR1*, включающий мутацию G961625T, приводящую к аминокислотной замене D1246Y, содержит 1 участок, соответствующий сайту рестрикции эндонуклеазы BglII. Поэтому в случае дикого генотипа *Plasmodium falciparum* исходный фрагмент дезоксирибонуклеиновой кислоты разрезается на 2 коротких участка (300 и 269 bp). При мутации D1246 замена нуклеотида G > T приводит к исчезновению сайта рестрикции, поэтому на электрофореграмме будет регистрироваться только 1 исходный фрагмент (509 bp). На основе анализа полученных данных разработаны критерии оценки лекарственной устойчивости *Plasmodium falciparum*. О снижении чувствительности к мефлохину и его производным возбудителей тропической малярии могут свидетельствовать положительные результаты, полученные при использовании разработанных методик выявления гаплотипов A > T (N86Y (бэнд 417 bp)), A > G (N1042D (бэнды 248, 99 и 25 bp)), G > T (D1246Y (509 bp)). Разработанные методики могут использоваться для анализа распространения лекарственно устойчивой тропической малярии.

Ключевые слова: тропическая малярия; *Plasmodium falciparum*; нуклеотидные полиморфизмы; рестрикционные фрагменты; полимеразная цепная реакция; специфические праймеры; амплификация; ампликон; миссенс-мутация.

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BACKGROUND

Drug resistance in parasites is associated with their high proliferative activity and the presence in the genome of variable regions susceptible to single nucleotide polymorphisms (SNPs) mutations. The development of drug resistance in plasmodia depends on the specific mechanism of pharmacological action of the antimalarial drug [1]. Plasmodia acquires resistance from mutations that change the structure of proteins, particularly enzymes that serve as targets for antiprotozoal drugs [2–4].

The development of plasmodium resistance to 4-aminoquinoline derivatives (mefloquine, quinine, and quinidine) is associated with mutations in *PFMDR1* gene (*Plasmodium falciparum* multidrug resistant-1), encoding the transport protein of the digestive vacuole membrane [5]. Modified proteins can excrete metabolites of antimalarial drugs, reducing their concentration and preventing the formation of toxic complexes [6–9].

The SNPs of the *PfMDR1* gene, such as A958146T (N86Y), A961013G (N1042D), and G961625T (D1246Y), are most often associated with resistance to mefloquine and its derivatives [10,11]. The A958146T mutation, which results in an amino acid N86Y substitution, is most common among African and Asian isolates of *P. falciparum*. It causes the drug resistance of plasmodia during monotherapy with mefloquine and along with other drugs [12]. Polymorphisms A961013G (N1042D) and G961625T (D1246Y) are strongly associated with mefloquine resistance and are most characteristic of the parasite population distributed in South America [13].

Recently, the restriction fragment length analysis method based on the polymerase chain reaction (PCR-RFLP) has often been used to identify the SNPs of malarial plasmodia [14]. This technique has high accuracy and specificity. In addition, unlike other methods for identifying gene mutations, such as Sanger sequencing, whole genome and whole exome sequencing PCR-RFLP is a substantial economical technique for screening studies [15]. However, the technique for its implementation has not yet been standardized; thus, there is a need to develop independent research modes. Practical experience shows that the representativeness of the results obtained largely depends on the equipment and reagents used, which makes it difficult to widely apply the technique in clinical practice [13]. Therefore, it is necessary to develop a method for identifying SNPs A958146T, A961013G, and G961625T in the gene *PfMDR1*, which lead to corresponding amino acid substitutions N86Y, N1042D, and D1246Y. This method would be suitable for assessing the resistance of tropical malaria pathogens to mefloquine and its derivatives.

This study aimed to develop a method to identify single-nucleotide polymorphisms of the *PfMDR1* gene (SNPs A958146T, A961013G, and G961625T) using PCR-RFLP analysis.

MATERIALS AND METHODS

Sample preparation. The DNA was isolated from the blood of patients after treating them with a phenol-chloroform mixture in accordance with the generally accepted procedure [16].

Primers for PCR were designed using the online BLAST tool based on the genome “*Plasmodium falciparum* 3D7 genome assembly, chromosome: 5”, taken from NCBI — NC 004326.2.

Restriction endonucleases. Optimal restriction enzymes for use in *P. falciparum* genotyping were selected *in-silico* using the databases of sequenced genomes with a program for simulating genome cleavage by restriction endonucleases.

DNA amplification. A Biorad C1000 amplifier was used for PCR. The reaction mixture included 5.2 µl of deionized water, 2 µl of 5x Taq Red buffer, 1.2 µl of 2.5-mM dNTPs (Evrogen, Russia), 0.2 µl of 20 µM each of the forward and reverse primers, 0.2 µl of Taq-DNA polymerase (SibEnzyme, Russia); 1 µl of 1 U of isolated DNA template. The number of amplification cycles and amplification conditions (initial denaturation, main denaturation, primer annealing, elongation, and final elongation) were individually selected.

Documentation of results. Amplification products were separated by electrophoresis on a 2% agarose gel using a Sub-Cell GT electrophoretic chamber (Biorad, USA). We used LE agarose (ThermoFisher) diluted in TBE buffer with the addition of ethidium bromide (biotechnology grade). The size of the amplified DNA regions was estimated using standard DNA length markers (100 and 50+ bp DNA Ladder). The electrophoresis results were recorded using an ECX-20M transilluminator (Vilber Lourmat, France). The results were recorded using a gel documentation system.

Clinical material. We studied blood samples obtained from patients with tropical malaria from various endemic regions (Africa, Southeast Asia, and Oceania) from the collection of the Department of Biology of the S.M. Kirov Military Medical Academy.

RESULTS AND DISCUSSION

The N86Y mutation is associated with a transversion of nucleotide 1 in codon 86 at position 958146 of the *PfMDR1* gene when replacing adenine with thymine (T > A (TAT > AAT)), which leads to replacing asparagine with tyrosine in the multidrug resistance polypeptide of *P. falciparum* [17]. To develop a method for identifying

the N86Y mutation, at stage 1, pairs of primers were selected to isolate parasitic DNA fragments, including the 86th codon. The main criteria for selection were specificity in relation to the genome of *P. falciparum*, absence of crossreactions with nucleotide sequences of human genomes and normal and pathological microflora. BLAST showed that these requirements were met by four pairs of primers, which allowed the amplification of fragments of length 948, 417, 106, and 105 bp (Table 1).

Primer pairs No. 3 and 4, which gave DNA sequences of 105 and 107 bp in length, respectively, were excluded from further development owing to the short length of the region they amplified, which can complicate the restriction analysis. For further analysis, we used primers of the pairs 1 and 2, which amplified fragments of 948 and 417 bp, respectively.

At stage 2, restriction enzymes were selected using the NEBcutter online tool to search for restriction sites in a given sequence. The main criteria were the inclusion of the sequence of the analyzed mutation in the restriction site, as well as the number of restriction points within

the amplicon analyzed. The results showed that the endonucleases *ApoI* and *MluCI* met these requirements. A comparative analysis of the number of restriction points within the analyzed amplicons showed that the 948-bp amplicon included 17 regions corresponding to the *MluCI* restriction site and the *ApoI* restriction site 3. The 417-bp amplicon included three *MluCI* restriction sites and one *ApoI* site (Table 2).

Based on the data obtained, the restriction enzyme *MluCI* was excluded from further analysis, as its use led to the division of the 948- and 417-bp amplicons into 18 and 8 parts, respectively, which would significantly complicate the analysis of the electropherogram during further analysis. Similarly, a pair of primers limiting the 948-bp amplicon, which, if treated with the restriction enzyme *ApoI*, would be divided into four fragments difficult to distinguish during electrophoretic separation (Fig. 1), was excluded from further analyses.

A fragment of the parasitic genome with a length of 417 bp with one point of application of the *ApoI* restriction enzyme was selected to identify N86Y (Fig. 2).

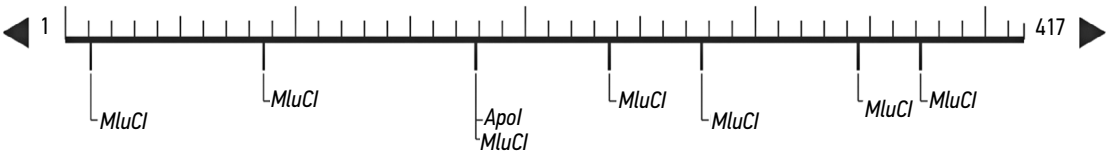


Fig. 1. Restriction points of *ApoI* amplicon 417 bp
Рис. 1. Точки рестрикции *ApoI* ампликона 417 bp

Table 1. Primers for specific amplification of *Plasmodium falciparum* genome fragments including the 86th codon
Таблица 1. Праймеры для специфической амплификации фрагментов генома *Plasmodium falciparum*, включающих 86-й кодон

№	Primer	Amplicon
1	Forward 5'-TGATTTTGTGTTGAAAGATGGGT-3' Reverse 5'-TGGGGTATTGATTCGTTGCAC-3'	948 bp
2	Forward 5'-AGAGTACCGCTGAATTATTAG-3' Reverse 5'-CCTGAACCTCACTGTTCTAAAT-3'	417 bp
3	Forward 5'-TGATTTTGTGTTGAAAGATGGGT-3' Reverse 5'-TCAGCGGTACTCTTTTGTTC A-3'	106 bp
4	Forward 5'-TGATTTTGTGTTGAAAGATGGGT-3' Reverse 5'-CAGCGGTACTCTTTTGTTC AACT-3'	105 bp

Table 2. Comparative characteristics of *MluCI* and *ApoI* restrictases with respect to amplicons 948 and 417 bp
Таблица 2. Сравнительная характеристика рестриктаз *MluCI* и *ApoI* по отношению к ампликонам 948 и 417 bp

Restrictase	Restriction site	Number of restriction sites	
		948-bp amplicon	417-bp amplicon
<i>MluCI</i>	5' ... ↑AATT ... 3' 3' ... TTAA↓ ... 5'	17	7
<i>ApoI</i>	5' ... R↑AATTY ... 3' 3' ... YT TAA↓R ... 5'	3	1

Note: ↑ and ↓ are restriction points..

5'-AGAGTACCGCTGAATTATTTAGAAAAATAAAGAATGAGAAAATATCATTTTTT
TTACCGTTTAAATGTTTACCTGCACAACATAGAAAATTATTATTTATATCATTG
TATGTGCTGTATTATCAGGAGGAACATTACCTTTTTTTATATCTGTGTTTGGTG
TAATATTAAGAAGACATGA/TATTAGGTGATGATTAATCCTATAATATTATCAT
TAGTATCTATAGGTTTAGTACAATTTATATTATCAATGATATCAAGTTATTGTAT
GGATGTAATTACATCAAAAATATTA AAAA AACTTTAAAGCTTGAATATTTAAGAA
GTGTTTTTTATCAAGATGGACAATTCATGATAATAATCCTGGATCTAAATTA
GATCTGATTAGATTTTTATTAGAACAAAGTGAGTTCAGG-3'

Fig. 2. Amplified fragment of the *PfMDR1* gene. The primers are highlighted in italics, the dot mutation in the 86th codon is in bold, the fragment containing the *ApoI* restriction site is underlined

Рис. 2. Амплифицированный фрагмент гена *PfMDR1*. Курсивом выделены праймеры, жирным шрифтом — точечная мутация в составе 86-го кодона, подчеркиванием — фрагмент, содержащий сайт рестрикции *ApoI*

The *ApoI* restriction endonuclease site includes codon 86 of the *PfMDR1* gene and exists only in the structure of the wild (unmodified) genotype of *P. falciparum* (Fig. 3).

Thus, with an unchanged *P. falciparum* genotype, the isolated DNA region would have been divided into two fragments of 239 and 179 bp. For an altered genotype containing the point mutation A958146T (N86Y), the original 417-bp *Plasmodium* fragment will be retained.

The system we constructed for detecting the N86Y mutation was tested by examining the genetic material isolated from blood samples of patients with tropical malaria containing *P. falciparum* trophozoites. The results were considered based on the analysis of electrophoretic separation of the amplification products on a 2% agarose gel after treating the amplicon with *ApoI* endonuclease. The examination of control samples indicated that no false-positive results were observed. The discriminatory ability of the developed method was revealed in relation to samples obtained from various parasitic strains (Fig. 4).

The N1042D mutation is associated with the transformation of nucleotide 1 in the codon 1042 at position 961013 of the *PfMDR1* gene from adenine to guanine (A > G (AAT > GAT)), which leads to the substitution of asparagine with aspartic acid in the multidrug resistance polypeptide of *P. falciparum* [17]. To select a region of the parasitic genome containing the mutation A961013G (N1042D) and suitable for restriction analysis, we selected only one pair of specific primers, namely, N1042D F: 5'-GCTCTTCTGGTTAGCATGGTT-3' (21 bp); N1042D R: 5'-AGGATCCAAACCAATAGGCAA-3' (21 bp). The fragment of parasitic DNA they amplify had a size of 404 bp in *Plasmodium*. Primers for other nucleotide sequences either did not have the necessary specificity for the human genome or demonstrated the possibility of crossreactions with known nucleotide sequences of normal or pathological microflora.

The chosen restriction enzyme to detect the N1042D mutation included sites of three endonucleases, namely, *AseI*, *MluCI*, and *MseI* (Table 3).

Endonuclease *AseI* had the smallest number of restriction points relative to the 404-bp amplicon.

5'-GAACATG.¹²ATTAGGTGATG-3'⁴

Fig. 3. Fragment of the *PfMDR1* gene site with the 86th codon and the *ApoI* restriction site: 1 — the restriction point when the *ApoI* endonuclease recognizes the restriction site as part of the *PfMDR1* gene; 2 — the 86th codon of the *PfMDR1* gene encoding asparagine (in this case wild type) or threonine in the polypeptide of the *Plasmodium falciparum* multiple resistance protein; 3 — the first nucleotide of the 86th codon (in this case, adenine), which determines the polymorphism of the *PfMDR1* gene on the basis of drug resistance to mefloquine; 4 — the restriction site of *ApoI* endonuclease

Рис. 3. Фрагмент участка гена *PfMDR1* с 86-м кодоном и сайтом рестрикции *ApoI*: 1 — точка рестрикции при распознавании эндонуклеазой *ApoI* сайта рестрикции в составе гена *PfMDR1*; 2 — 86-й кодон гена *PfMDR1*, кодирующий аспарагин (в данном случае диккий тип) или треонин в полипептиде белка множественной резистентности *Plasmodium falciparum*; 3 — первый нуклеотид 86-го кодона (в данном случае аденин), определяющий полиморфность гена *PfMDR1* по признаку лекарственной устойчивости к мефлохину; 4 — сайт рестрикции эндонуклеазы *ApoI*

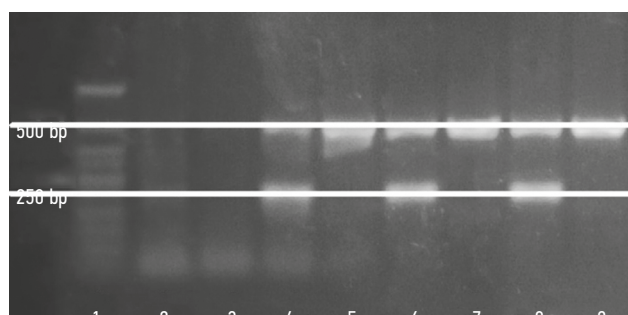


Fig. 4. Electrophoregram of the results of PCR-RFLP mutation N86Y in the *PfMDR1* gene using *ApoI*: 1 — length marker; 2, 3 — negative control; 4 — 86Y; 5, 6 — 86Y/N; 7 — 86N

Рис. 4. Электрофореграмма результатов PCR — RFLP-мутации N86Y в гене *PfMDR1* с помощью *ApoI*: 1 — маркер длин; 2, 3 — отрицательный контроль; 4 — 86Y; 5, 6 — 86Y/N; 7 — 86N

Processing the analyzed fragment of parasitic DNA revealed four clearly distinguishable bands on the electropherogram (Fig. 5).

To detect A961013G (N1042D), a 404-bp long fragment of the parasitic genome with three application points of the *AseI* restriction enzyme was selected (Fig. 6).

Таблица 3. Сравнительная характеристика рестриктаз *AseI*, *MluCI* и *MseI* по отношению к ампликону 404 bp

Note: $\uparrow$ and $\downarrow$ are restriction points.

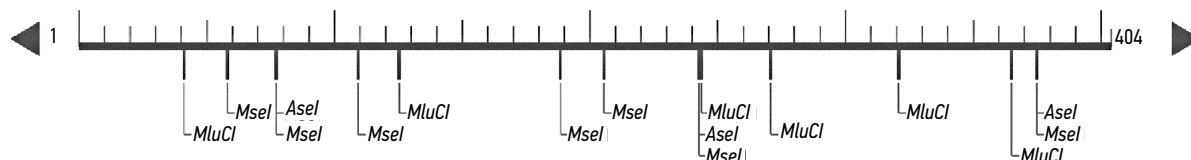


Fig. 5. Restriction points of *AseI* amplicon 404 bp
Рис. 5. Точки рестрикции *AseI* ампликона 404 bp

5'-TATGTCAGCGGAGTTTTTGCATTTAGTTCAGATGATGAAATGT
TTAAAGATCCAAGTTTTTAAATACAGGAAGCATTTTATAATATGC
ATACTGTTAT↑↓TAATTATGGTTTAGAAGATTATTTCTGTAATTTG
ATAGAAAAAGCTATTGATTATAAAAATAAAGGACAAAAAAGAA
GAATTATTGTAAATGCAGCTTTATGGGGATTTCAGTCAAAGCGCT
CAATTATTTAT↑↓TAATAGTTTTGCCTATTGGTTTGGATCCTTCTT
AATTAAGAGAGGTACTATATTAGTTGATGACTTTATGAAATCCTT
ATTTACTTTTATATTTACTGGTAGTTATGCTGGAAAAAT↑↓TAATGT
CCTTAAAGGAGATTTCAGA-3'

Fig. 6. Amplified fragment of the *PfMDR1* gene. Primers are highlighted in italics, restriction sites are in bold, DNA fragment incision points (restriction points) are indicated with arrows, and codon 1042 is underlined

Рис. 6. Амплифицированный фрагмент гена *PJMDR1*. Курсивом выделены праймеры, жирным шрифтом — сайты рестрикции, стрелками — места разреза фрагмента ДНК (точки рестрикции), подчеркиванием — кодон 1042

5'...TTAT...¹TA²ATAGTTT...3'

Fig. 7. Fragment of a section of the *PfMDR1* gene with the 1042nd codon and the *Asel* restriction site: 1 — the restriction point when the *Asel* endonuclease recognizes the restriction site as part of the *PfMDR1* gene; 2 — the 1042 codon of the *PfMDR1* gene encoding asparagine (in this case wild type) or aspartic acid in the *Plasmodium falciparum* multiple resistance polypeptide; 3 — the first nucleotide of codon 1042 (in this case adenine), which determines the polymorphism of the *PfMDR1* gene on the basis of drug resistance to mefloquine; 4 — *Asel* endonuclease restriction site

Рис. 7. Фрагмент участка гена *PfMDR1* с 1042-м кодоном и сайтом рестрикции *Asel*: 1 — точка рестрикции при распознавании эндонуклеазой *Asel* сайта рестрикции в составе гена *PfMDR1*; 2 — 1042 кодон гена *PfMDR1*, кодирующий аспарагин (в данном случае дикий тип) или аспарагиновую кислоту в полипептиде множественной резистентности *Plasmodium falciparum*; 3 — первый нуклеотид 1042 кодона (в данном случае аденин), определяющий полиморфность гена *PfMDR1* по признаку лекарственной устойчивости к мефлохину; 4 — сайт рестрикции эндонуклеазы *Asel*

The *AseI* restriction endonuclease site included codon 1042 of the *PfMDR1* gene and occurred only in the wild (unmodified) genotype of *P. falciparum* (Fig. 7).

The amplified fragment of the *PfMDR1* gene contained three regions corresponding to the *Asel* restriction site, one of which includes the sequences of codon 1042. Therefore, in the case of the *Plasmodium* wild genotype, the original DNA fragment (*PfMDR1* 404 bp) was cut into four short sections (132, 116, 99, and 25 bp). Because of a mutation in the codon 1042, the nucleotide substitution led to the disappearance of the central restriction site; thus, the electropherogram recorded three or two fragments (248, 99, and 25 or 248 and 99 bp).

Testing of the constructed system for detecting the N1042D mutation on clinical material showed no false-positive results when examining control samples. The developed method could distinguish *P. falciparum* strains isolated from the blood of different patients, which,

if confirmed by clinical cases, suggests drug resistance of parasites (Fig. 8).

The D1246Y mutation is associated with a G > T transversion (GAT > TAT) of the nucleotide 1 in codon 1246 at position 961625 of the *PFMDR1* gene from guanine to thymine, which leads to the replacement of aspartic acid with tyrosine in the multidrug resistance polypeptide embedded in the membrane of the trophozoite digestive vacuole of *P. falciparum* [17].

During the construction of the system for detecting the D1246Y mutation, two pairs of primers were used to isolate parasitic DNA fragments, including codon 1246. The lengths of the amplified fragments of parasitic DNA were 732 and 509 bp (Table 4).

A search for restriction sites in a given sequence showed that five endonucleases, namely, *Sau3AI*, *Mbol*, *DpnII*, *BstYI*, and *BglII*, can be used to detect the N1042D mutation (Table 5).

The *BglII* restriction enzyme, which has one restriction site in the nucleotide sequence of each of the selected amplicons, was selected. Further developments used a short fragment of 509 bp (Fig. 9).

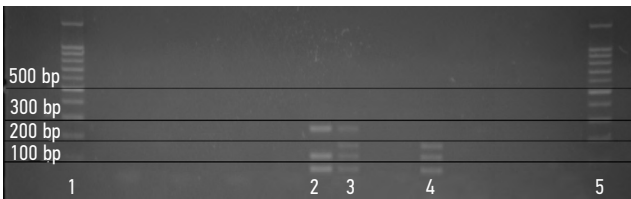


Fig. 8. Electrophoregram of PCR-RFLP results for detecting the N1042D mutation in the *PfMDR1* gene using *AseI*: 1.5 — length marker; 2 — 1042D; 3 — 1042N/D; 4 — N1042

Рис. 8. Электрофореграмма результатов PCR-RFLP по выявлению мутации N1042D в гене *PfMDR1* с помощью *AseI*: 1,5 — маркер длин; 2 — 1042D; 3 — 1042N/D; 4 — N1042

To detect the single nucleotide polymorphism G961625T (N1042D), a 509-bp fragment of the parasitic genome with one restriction site for the *BglII* endonuclease was selected (Fig. 10).

The *BglII* restriction endonuclease site included codon 1246 of the *PfMDR1* gene and occurred only in the structure of the wild (unmodified) genotype of *P. falciparum* (Fig. 11).

The amplified fragment of the *PfMDR1* gene contains one region corresponding to the *BglII* restriction site, which includes the sequence of codon 1246. Therefore, in the case of

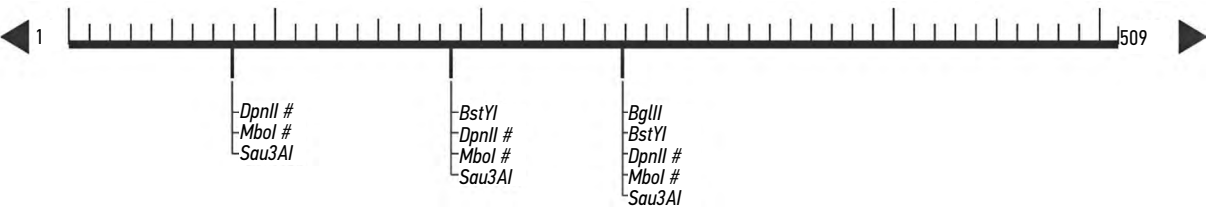


Fig. 9. Restriction points of *BglII* amplicon 509 bp
Рис. 9. Точки рестрикции *BglII* ампликона 509 bp

Table 4. Parameters for specific amplification of *Plasmodium falciparum* genome fragments including the 1246th codon
Таблица 4. Параймеры для специфической амплификации фрагментов генома *Plasmodium falciparum*, включающих 1246-й кодон

№	Primer	Fragment length
1	F: 5'-CTACAGCAATCGTTGGAGAAA-3' R: 5'-GAGAATAGCTATAGCTAGAGC-3'	509
2	F: 5'-CAGCAATCGTTGGAGAAACAGG-3' R: 5'-CGTGTGTTCCATGTGACTGT-3'	732

Table 5. Comparative characteristics of restrictases *Sau3AI*, *Mbol*, *DpnII*, *BstYI* and *BglII* with respect to amplicons 509 and 732 bp
Таблица 5. Сравнительная характеристика рестриктаз *Sau3AI*, *Mbol*, *DpnII*, *BstYI* и *BglII* по отношению к ампликонам 509 и 732 bp

Restrictase	Restriction site	Number of restriction sites	
		509-bp amplicon	732-bp amplicon
<i>Sau3AI</i>	5' ... ↑GATC ... 3' 3' ... CTAG↓ ... 5'	3	5
<i>Mbol</i>	5' ... ↑GATC ... 3' 3' ... CTAG↓ ... 5'	3	5
<i>DpnII</i>	5' ... ↑GATC ... 3' 3' ... CTAG↓ ... 5'	3	5
<i>BstYI</i>	5' ... R↑GATCY ... 3' 3' ... YCTAG↓R ... 5'	2	2
<i>BglII</i>	5' ... A↑GATCT ... 3' 3' ... TCTAG↓A ... 5'	1	1

5'-CTACAGCAATCGTTGGAGAAACAGGTAGTGGAAAATCA
 ACTTTTATGAATCTCTTATTAAGATTTTATGACTTGAAAAA
 TGATCACATTATATTAATAAATGATATGACAAATTTTCAAG
 ATTATCAAAATAATAATAAATTCATTGGTTTTAAAAAAT
 GTAAATGAATTTTCAAACCAATCTGGATCTGCAGAAGATT
 ATACTGTATTTAATAATAATGGAGAAATATTATTAGATGATA
 TTAATATATGTGATTATAACTTAAGA(G/D)ATCTTAGAACT
 TATTTTCAATAGTTAGTCAAGAACCCATGTTATTTAATATG
 TCCATATATGAAAATATCAAATTTGGAAGAGAAGATGCAA
 CATTGGAAGATGTTAAACGTGTTAGTAAGTTTGCTGCTAT
 AGATGAATTTATCGAATCATTACCAAATAAATATGATACAA
 ATGTTGGACCATATGGTAAAAGCTTATCAGGTGGACAAAA
 ACAGAGAATAGCTATAGCTAGAGC-3'

Fig. 10. Amplified fragment of the *PfMDR1* gene. Primers are highlighted in italics, and codon 1246 is underlined

Рис. 10. Амплифицированный фрагмент гена *PfMDR1*. Курсивом выделены праймеры, подчеркиванием — кодон 1246

5'...AAGA.↓.GATCTTA...3'

Fig. 11. Fragment of the *PfMDR1* gene site with the 1246th codon and the *BglII* restriction site: 1 — the restriction point when the *BglII* endonuclease recognizes the restriction site as part of the *PfMDR1* gene; 2 — the 1246th codon of the *PfMDR1* gene encoding aspartic acid (in this case wild type) or tyrosine in the *PLASMODIUM* multiple resistance protein polypeptide falciparum; 3 — the first nucleotide of the 1246th codon (in this case, adenine), which determines the variant of the *PfMDR1* gene on the basis of drug resistance to mefloquine; 4 — the site of restriction of endonuclease *BglII*

Рис. 11. Фрагмент участка гена *PfMDR1* с 1246-м кодоном и сайтом рестрикции *BglII*: 1 — точка рестрикции при распознавании эндонуклеазой *BglII* сайта рестрикции в составе гена *PfMDR1*; 2 — 1246-й кодон гена *PfMDR1*, кодирующий аспарагиновую кислоту (в данном случае дикий тип) или тирозин в полипептиде белка множественной резистентности *PLASMODIUM falciparum*; 3 — первый нуклеотид 1246-го кодона (в данном случае аденин), определяющий вариант гена *PfMDR1* по признаку лекарственной устойчивости к мефлохину; 4 — сайт рестрикции эндонуклеазы *BglII*

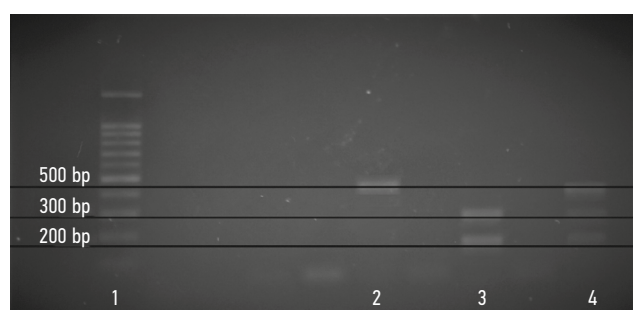


Fig. 12. Electrophoregram of PCR-RFLP results for detecting the D1246Y mutation in the *PfMDR1* gene using *AseI*: 1 — length marker; 2 — 1246Y; 3 — 1246 D; 4 — 1246.D/Y

Рис. 12. Электрофореграмма результатов PCR-RFLP по выявлению мутации D1246Y в гене *PfMDR1* с помощью *AseI*: 1 — маркер длин; 2 — 1246Y; 3 — 1246 D; 4 — 1246.D/Y

the wild genotype of *P. falciparum*, the original DNA fragment (569 bp of *PfMDR1*) was cut into two short sections (300 and 269 bp). With a mutation in the codon 1246, the nucleotide replacement led to the disappearance of the restriction site; thus, only one original fragment (509 bp) was recorded on the electropherogram.

When examined using a constructed system of blood samples containing pathogens of tropical malaria, differences in results were revealed, indicating the technique can discriminate mutation D1246Y (Fig. 12).

The data were to develop criteria for assessing and interpreting the results of examination of biological samples containing genetic material of tropical malaria pathogens. The detection of amplicons of 417, 239, and 179 bp in size indicated the presence of DNA fragments, including the mutant allele A > T (N86Y) of the *PfMDR1* gene in the sample of *P. falciparum* (Fig. 13).

Moreover, the appearance of the 417-bp band on the electropherogram indicates the presence of the A > T (N86Y) mutation in the *P. falciparum* genotype and its possible resistance to mefloquine and its derivatives. The 239- and 179-bp amplicons are the sign of the wild genotype of *P. falciparum* and the drug sensitivity of the parasites. The simultaneous appearance of three bands (417, 239, and 179 bp) on the electropherogram suggests a mixed infection and the presence of both sensitive and drug-resistant *P. falciparum* in the blood sample. The remaining electrophoresis options should be regarded as insignificant, which requires a repeat study.

The separation of amplicon restriction fragments by electrophoresis, including SNP A > G (N1042D) of the *PfMDR1* gene, showed that the detection of bands of 248, 132, 116, 99, and 25 bp should be considered a positive result (Fig. 14).

Moreover, the appearance of bands 132, 116, 99, and 25 bp during electrophoretic dilution of restriction products

indicates the presence of the wild (unchanged) genotype of *P. falciparum* and the preservation of drug sensitivity of the pathogen of tropical malaria. A positive electrophoresis result with bands of sizes 248, 99, and 25 bp is a sign of the A > G (N1042D) mutation and drug resistance of the pathogens. The appearance of bands 248, 132, 116, 99, and 25 bp in one sample suggests the persistence of various parasitic strains, including those sensitive to antimalarial drugs and those with resistant to drugs. If there were no positive results, there would be a need to repeat the study.

When examining blood samples for the presence of the G > T (D1246Y) mutation of the *PfMDR1* gene of *P. falciparum*, detection of bands of 300, 209, and 509 bp is considered a positive result (Fig. 15).

The appearance of 300- and 209-bp bands upon electrophoretic dilution of restriction products indicates the presence of the wild (unchanged) genotype of *P. falciparum* and the preservation of drug sensitivity of the parasites. A positive electrophoresis result with 509-bp amplicons is a sign of the presence of the D1246Y mutation and drug resistance of the pathogens. The appearance of 300-, 209-, and 509-bp bands suggests persistence of various parasitic

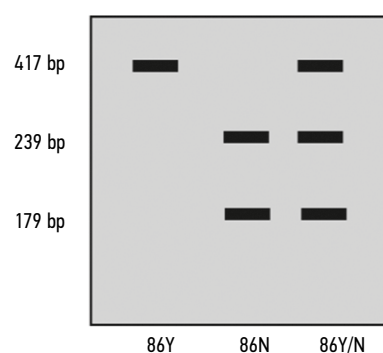


Fig. 13. Schematic structure of PCR-RFLP cleavage of fragment 86 by *Apol* restrictase: 86Y — tyrosine (absence of restriction, single band 417 bp, sign of *Plasmodium falciparum* resistance); 86N — asparagine (restriction, bands 239 and 179 bp, sign of absence of *Plasmodium falciparum* resistance); 86Y/N — tyrosine/asparagine (bands 417, 239 and 179 bp, persistence of wild and drug-resistant strains of *Plasmodium falciparum*)

Рис. 13. Схематическая структура PCR-RFLP расщепление фрагмента 86 с помощью рестриктазы *Apol*: 86Y — тирозин (отсутствие рестрикции, одиночный бэнд 417 bp, признак резистентности *Plasmodium falciparum*); 86N — аспарагин (рестрикция, бэнды 239 и 179 bp, признак отсутствия резистентности *Plasmodium falciparum*); 86Y/N — тирозин/аспарагин (бэнды 417, 239 и 179 bp, персистирование диких и лекарственно устойчивых штаммов *Plasmodium falciparum*)

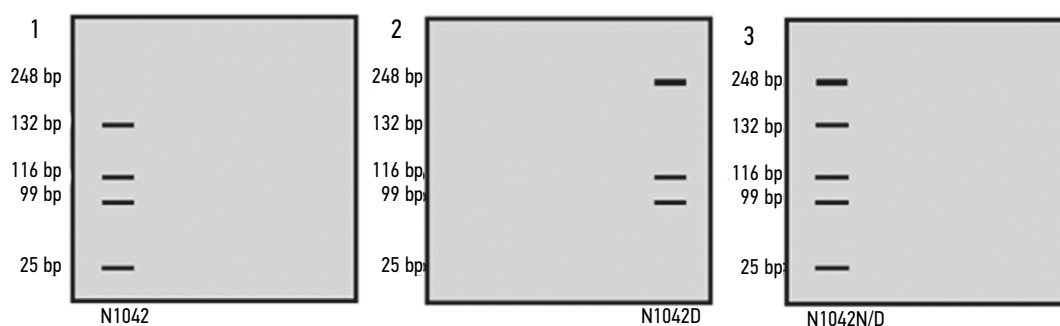


Fig. 14. Schematic structure of electrophoretic dilution of the amplified fragment using *AseI*: 1 — *PfMDR1* N1042 — AAT (asn), wild (unchanged) type genotype, a sign of *Plasmodium falciparum* drug sensitivity; 2 — *PfMDR1* 1042D — GAT (asp), a genotype containing the N1042D mutation, a sign of *Plasmodium falciparum* drug resistance; 3 — persistence of wild and drug-resistant strains of *Plasmodium falciparum*

Рис. 14. Схематическая структура электрофоретического разведения амплифицированного фрагмента при использовании *AseI*: 1 — *PfMDR1* N1042 — AAT (асн), генотип дикого (неизмененного) типа, признак лекарственной чувствительности *Plasmodium falciparum*; 2 — *PfMDR1* 1042D — GAT (асп), генотип, содержащий мутацию N1042D, признак лекарственной резистентности *Plasmodium falciparum*; 3 — персистенция диких и лекарственно устойчивых штаммов *Plasmodium falciparum*

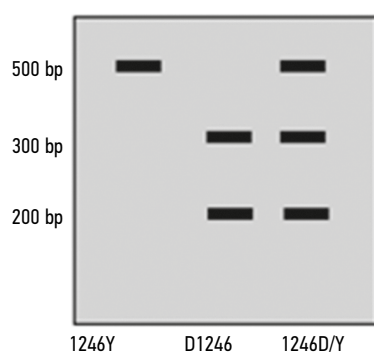


Fig. 15. Schematic structure of electrophoretic dilution of the amplified fragment using *BglII*: 1 — *PfMDR1* D1246 — GAT (asp) — wild (unchanged) type genotype, a sign of *Plasmodium falciparum* drug sensitivity; 2 — *PfMDR1* 1246Y — TAT (dash), a genotype containing the D1246Y mutation, a sign of *Plasmodium falciparum* drug resistance; 3 — persistence of wild and drug-resistant strains of *Plasmodium falciparum*

Рис. 15. Схематическая структура электрофоретического разведения амплифицированного фрагмента при использовании *BglII*: 1 — *PfMDR1* D1246 — GAT (асп) — генотип дикого (неизмененного) типа, признак лекарственной чувствительности *Plasmodium falciparum*; 2 — *PfMDR1* 1246Y — TAT (тир), генотип, содержащий мутацию D1246Y, признак лекарственной резистентности *Plasmodium falciparum*; 3 — персистенция диких и лекарственно устойчивых штаммов *Plasmodium falciparum*

strains, including those sensitive to antimalarial drugs and those resistant to drugs. If there are no positive results, a repeated study is required.

CONCLUSION

Our findings indicate that the developed methods have a discriminatory ability against various genotypes of tropical malaria pathogens and can be used to genotype *P. falciparum* in blood samples of patients with tropical malaria. The genetic markers of *P. falciparum* with reduced sensitivity

to mefloquine and its derivatives are *Plasmodium* genotypes containing SNPs A958146T, A961013G, and G961625T, leading to the corresponding amino acid substitutions N86Y, N1042D, and D1246Y of the *PfMDR1* gene. The positive results obtained using the methods developed in this study for identifying haplotypes N86Y (band 417 bp), N1042D (bands 248, 99, and 25 bp), and D1246Y (509 bp) indicate a decrease in the sensitivity of tropical malaria pathogens to mefloquine and its derivatives. The developed methods can be used to analyze the spread of tropical malaria pathogens resistant to mefloquine and its derivatives.

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