JBACHS
Journal of Basic and Clinical Health Sciences

J Basic Clin Health Sci 2020; 4:154-159
https://doi.org/10.30621/jbachs.2020.958

Original investigation

Comparison of Different 18S rRNA Primers with Conventional PCR Methods in Determination of *Plasmodium spp.* and Evaluation of Nested PCR Method

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Received: 2019-07-06; **Accepted:** -2020-03-17; **Available Online Date:** 15.05.2020

Cite this article as: Usluca S, Celebi B. Comparison of Different 18S rRNA Primers with Conventional PCR Methods in Determination of Plasmodium spp. and Evaluation of Nested PCR Method. J Basic Clin Health Sci 2020; 4:154-159.

ABSTRACT

Purpose: In this study, our objective was to develop a conventional PCR method in our laboratory to support microscopy and rapid diagnostic tests in routine diagnosis of malaria. For this purpose, by comparing two different primer sets, it was aimed to determine which primer set gave better results in diagnosis and to use this primer set in future studies.

Methods: Microscopic examination is the gold standard for diagnosis of *Plasmodium* infections. The sensitivity and specificity of conventional PCR method, which was based on two different primer sets with a common gene region, were calculated. Afterward, nested PCR were performed for species differentiation using PCR products obtained with both primer pairs.

Results: 165 of 168 blood samples (98.21%), which were microscopically *Plasmodium vivax* positive, were also positive with rPLU1, rPLU5 primers. Furthermore, 163 of these samples (97.02%) were also positive with rPLU5, rPLU6 primers.

In addition, to evaluate whether the method detected all species, PCR was carried out with all species positive samples for both primer pairs. Comparison with microscopic examination showed that sensitivity and specificity of rPLU1 and rPLU5 primer pairs were 98.21% and 100%, respectively while sensitivity and specificity of rPLU5 and rPLU6 primer pairs were 97.02% and 100%, respectively. We found a perfect consistency between microscopy and PCR results with both primer sets.

Conclusion: Although there was no significant difference between two primer pairs, which provided better results for cases required a conventional first step PCR method during routine laboratory practice, we decided to prefer rPLU1 and rPLU5 primer pair.

Keywords: Conventional PCR, nested PCR, *Plasmodium spp.*, 18S rRNA primers

INTRODUCTION

Malaria is an important public health concern and is responsible worldwide for approximately 600.000 deaths annually (1). There are five species of *Plasmodium* which cause malaria infection in humans; *Plasmodium vivax* (*P. vivax*), *Plasmodium falciparum* (*P. falciparum*), *Plasmodium malariae* (*P. malariae*), *Plasmodium ovale* (*P. ovale*), and *Plasmodium knowlesi* (*P. knowlesi*) (2). While infections caused by *P. falciparum* has the highest mortality rate, *P. vivax* infections have a mild clinical course with a relatively low mortality rate (3). Malaria begun to spread again to regions like Eastern Europe and Central Asia, where it had been previously eradicated. Depending on increased population density to endemic regions through touristic activity and increased migration activities due to the wars and socioeconomic factors, the rate of imported malaria cases increased significantly. The mortality rate increased also from 3.8% up to 20%. Mainly delayed and misdiagnosis was blamed for this increase. Regarding the diagnosis, microscopic examination of thick and thin smear with Giemsa stain is gold standard. However, alternative diagnosis methods are also used, as microscopic examination depends on experienced microscopists. The easiest among these methods is rapid diagnostic tests, which do not require experienced technical personnel. Currently, molecular methods are also widely used for detection of mixed infections, which may be frequently overlooked with conventional methods (4). Determination of asymptomatic parasite carriers, who are the main reservoir of the parasite, is very important (1). Although molecular methods are highly sensitive, the sensitivity and specificity may change depending on targeted gene loci. Particularly cases with low parasitemia are the main problem in diagnosis of the disease.

Development of rapid, highly sensitive and specific, inexpensive and widely available diagnostic tests is crucial for fight against

the disease. In this context, comparative studies focused on the existing diagnostic methods are needed for determination of the most effective methods (5).

Since our laboratory is a National Malaria Reference Laboratory, rapid diagnostic test and molecular methods are used together, especially microscopy, which is gold standard method in diagnosis of malaria, in order not to misdiagnosis of the disease. While using commercial kits for molecular methods increases cost considerably, cost decreases considerably when using in-house methods. Our aim in this study is to develop a conventional PCR method in our laboratory to support microscopy and rapid diagnostic tests in routine diagnosis of malaria. For this purpose, by comparing two different primer sets, it was aimed to determine which primer set gave better results in diagnosis and to use this primer set in future studies.

METHODS

Because of our laboratory is Malaria Reference Laboratory, 168 whole blood samples were sent to our laboratory between 01/08/2012 and 01/08/2013 to confirm the diagnosis of malaria from Savur district of Mardin province. Confirmation studies of malaria were carried out with DNA samples obtained from blood samples. This study is a retrospective study using DNA samples stored at -20°C as replicate samples. In our study, microscopy results, which are the gold standard method in the diagnosis of malaria, were accepted as diagnostic criteria. Accordingly, samples evaluated as positive by microscopy were considered malaria positive, and samples that were evaluated as negative by microscopy were considered malaria negative.

It was reported that 18S rRNA gene-based primers had high sensitivity in diagnosis of malaria (6). In our study, we accepted microscopic examination as a gold standard and sensitivity and specificity of conventional PCR method, which was performed with two different 18S rRNA based primer sets (rPLU1, rPLU5 and rPLU5, rPLU6), were calculated. Then nested PCR was performed with usage of PCR product and species differentiation of *Plasmodium* was performed. A gradient PCR was carried out for optimization of nested PCR method and annealing temperature and $MgCl_2$ concentration suitable for amplification were determined.

According to the results of microscopic examination, 168 of 266 blood samples were positive and 98 were negative for *P. vivax*. We did not detect any mixed infection. Absence of parasites in a total of 200 microscope fields was defined as a negative result. DNA extraction was carried out in these samples with a commercial kit (QIAamp DNA mini kit, Germany) according to the manufacturer's recommendations. 200 µl blood sample was used for DNA extraction and the obtained 100 µl DNA was analyzed with conventional PCR method using two different primer sets (rPLU1, rPLU5 and rPLU5, rPLU6 primer sets) (7) (Table 1). The product of amplification was visualized in 1.5% agarose gel. A band of 100 bp was used as DNA marker.

Table 1. Primary sequences of target gene regions (7)

Primers	Sequences	Size
rPLU1	5′-TCA AAG ATT AAG CCA TGC AAG TGA-3′	1670 bp
rPLU5	5′-CCT GTT GTT GCC TTA AAC TCC-3′	
rPLU1	5′-TCA AAG ATT AAG CCA TGC AAG TGA-3′	1200 bp
rPLU6	5′-TTA AAA TTG TTG CAG TTA AAA CG-3′	
rVIV1	5′-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3′	117 bp
rVIV2	5′-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3′	
rFAL1	5′-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3′	205 bp
rFAL2	5′-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3′	
rMAL1	5′-ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC-3′	144 bp
rMAL2	5′-AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA-3′	
rOVA1	5′-ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA-3′	787 bp
rOVA2	5′-GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG-3′	

The PCR mixture was prepared with rPLU1, rPLU5 primers; 5 µl Platinum Taq buffer (Invitrogen, Brazil), 2 µl (50 mM) $MgCl_2$, 1 µl (10 mM) dNTP, 1 µl (10 pmol/µl) rPLU1, 1 µl (10 pmol/µl) rPLU5, 0.25 µl Platinum Taq polymerase enzyme (Invitrogen, Brazil), and a 5 µl DNA sample. Total volume was adjusted to 50 µl. The amplification started with an initial denaturation at 95°C for 5 minutes and then continued with 35 cycles at 95°C for 30 seconds, at 55°C for 1 minute and at 72°C for 1 minute and then final extension at 72°C for 5 minutes.

The PCR mixture was prepared with rPLU5, rPLU6 primers; 5 µl Platinum Taq buffer (Invitrogen, Brazil), 2 µl (50 mM) $MgCl_2$, 1 µl (10 mM) dNTP, 1 µl (10 pmol/µl) rPLU5, 1 µl (10 pmol/µl) rPLU6, 0.25 µl Platinum Taq polymerase enzyme (Invitrogen, Brazil), and 5 µl DNA sample and total volume was adjusted to 50 µl. Amplification was started with an initial denaturation at 95°C for 5 minutes and then continued with 35 cycles at 95°C for 30 seconds, at 55°C for 1 minute and at 72°C for 1 minute and then final extension at 72°C for 5 minutes. PCR amplification products were evaluated on 1.5% agarose gel.

The second step PCR reaction was carried out with PCR product obtained from both rPLU1, rPLU5 and rPLU5, rPLU6 primers using 1 µl from each primer for *P. vivax, P. falciparum, P. malariae,* and *P. ovale* and was processed as a separate reaction. A PCR mixture was prepared from 5 µl Platinum Taq buffer (Invitrogen, Brazil), 1.5 µl (50 mM) $MgCl_2$, 0.5 µl (10 mM) dNTP and 1 µl of rVIV1, rVIV2, rFAL1, rFAL2, rMAL1, rMAL2, rOVA1, rOVA2 primers, 0.25 µl Platinum Taq polymerase enzyme (Invitrogen, Brazil), and 2 µl first step PCR amplification product and volume was adjusted to 50 µl. The amplification was started with an initial denaturation at 95°C for 5 minutes, and then continued with 35 cycles at 95°C for 30 seconds, at 55°C for 1 minute and at 72°C for 1 minute and then final extension at 72°C for 5 minutes. After preparation of a 1.5% agarose gel, gel electrophoresis was run with 100 bp marker, positive and negative samples.

Gradient PCR was performed with optimization studies belonging to each PCR reaction based on each primer pairs, various $MgCl_2$ concentrations (1.5–4 mM) and annealing temperatures (48–60°C). The mixture densities and amplification conditions, which provided the best band images, were determined.

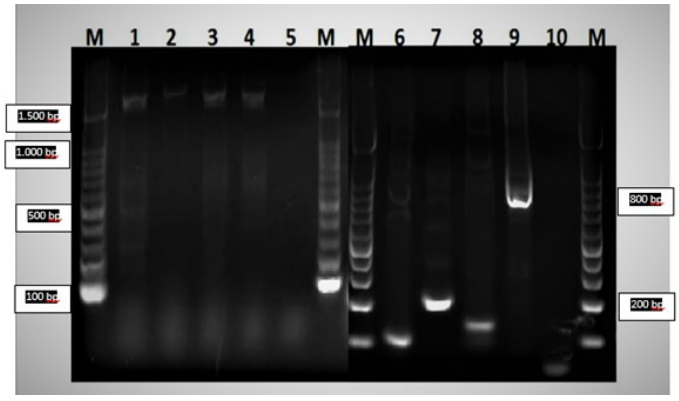

Figure 1. Image of conventional PCR products with rPLU1, rPLU5 primers and nested PCR products with species specific primers. **M:** Marker (100 bp), **1-4:** *Plasmodium* spp. positive samples (1.650 bp), **5:** *Plasmodium* spp. negative samples, **6:** *P. vivax* positive sample (117 bp), **7:** *P. falciparum* positive sample (205 bp), **8:** *P. malariae* positive sample (144 bp), **9:** *P. ovale* positive sample (787 bp), **10:** *Plasmodium* spp. negative sample.

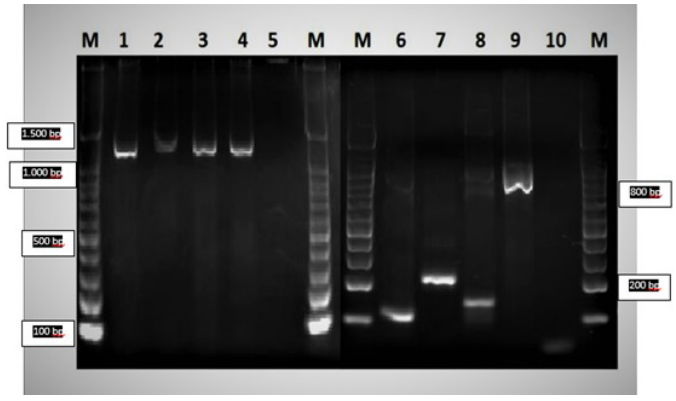

Figure 2. Image of conventional PCR products with rPLU5, rPLU6 primers and nested PCR products with species specific primers. **M:** Marker (100 bp), **1-4:** *Plasmodium* spp. positive samples (1.200 bp), **5:** *Plasmodium* spp. negative samples, **6:** *P. vivax* positive sample sample (117 bp), **7:** *P. falciparum* positive sample (205 bp), **8:** *P. malariae* positive sample (144 bp), **9:** *P. ovale* positive sample (787 bp), **10:** *Plasmodium* spp. negative sample.

This study was approved by the ethics committee of the Ankara Training and Research Hospital (16.01.2020–158/2019) and it was conducted according to the principles of the Declaration of Helsinki.

RESULTS

According to the microscopic examination 165 of 168 blood samples (98.21%), which were *P. vivax* positive samples were also positive with rPLU1 and rPLU5 primers (Figure 1). 163 of these samples (97.02%) were also positive with rPLU5 and rPLU6 primers (Figure 2). The study results were summarized in Table 2.

In addition, to evaluate whether the method detected all species, PCR was carried out with *P. falciparum, P. malariae,* and *P. ovale* positive samples for both primer pairs. Then the second step PCR reaction was carried out and 117 bp, 205 bp, 144 bp, and 787 bp bands were determined for *P. vivax, P. falciparum, P. malariae* and *P. ovale* positive samples, respectively (Figure 1, Figure 2). Comparison with microscopic examination showed that sensitivity and specificity of rPLU1, rPLU5 primer pair were 98.21% and 100% respectively, and sensitivity and specificity of rPLU5, rPLU6 primer pair were 97.02% and 100% respectively. At the end of our study, we decided to use nested PCR method in verification of *Plasmodium* species as gold standard.

Table 2. Comparison of microscopy and PCR results

		Microscopy Results			
		Positive	**Negative**	**Total**	**Kappa value**
PCR Results with rPLU1, rPLU5 primers	**Positive**	165	0	165	0.976
	Negative	3	98	101	
	Total	168	98	266	
PCR Results with rPLU5, rPLU6 primers	**Positive**	163	0	163	0.960
	Negative	5	98	103	
	Total	168	98	266	

We found a perfect consistency between microscopy and PCR results with both primer sets. While using rPLU1 and rPLU5 primers κ value of PCR results is determined 0.976 (0.949–1.000), using rPLU5 and rPLU6 primers κ value of PCR results is determined 0.960 (0.925–0.995) (8). Kappa value (κ) is a measure of fit that corrects the chance part of the compliance between two observers. Assessments are considered reliable if there is a high agreement between the two tests compared (9). The evaluation criteria of kappa value are specified below;

Kappa <0: No agreement

Kappa between 0.00 and 0.20: Slight agreement

Kappa between 0.21 and 0.40: Fair agreement

Kappa between 0.41 and 0.60: Moderate agreement

Kappa between 0.61 and 0.80: Substantial agreement

Kappa between 0.81 and 1.00: Almost perfect agreement (10).

The Tm values of the rVIV1, rVIV2, rFAL1, rFAL2, rMAL1, rMAL2, rOVA1, rOVA2 primers used in our study was 58.3°C, 63.4°C, 54.4°C, 61.5°C, 56°C, 51.5°C, 53.5°C and 56.7°C, respectively.

Regarding the optimization of nested PCR, a gradient PCR was carried out, in which temperatures between 50–60°C (51, 53, 54.5, 56 and 58°C) tried for determination of suitable annealing temperature. The gradient study is an amplification reaction, in which different temperatures are used in each column of thermal cycler for determination of optimum annealing temperature of PCR reaction. Thus, annealing temperature most suitable for the primers is determined with only one reaction. As a result of the visualization of PCR products, the temperature without nonspecific band was considered as optimum annealing temperature.

In the optimization studies belonging to each PCR reaction based on each primer pair, the best amplification product visualization was obtained at an annealing temperature of 55°C for rPLU1,

Figure 3. Target gene regions.

rPLU5 primers, at 52°C for rPLU5, rPLU6 primers and at 2 mM $MgCl_2$ concentration for both primer sets, while 1.5 mM $MgCL_2$ concentration and an annealing temperature of 58°C provided the best amplification product image in second step PCR reactions.

The primer set rPLU1 and rPLU5 have a larger gene sequence, while the primer set rPLU5 and rPLU6 represent a smaller region within the same gene sequence. Target gene regions are schematized in Figure 3. Since the rPLU1 and rPLU5 primer set could detect a larger gene sequence than the rPLU5 and rPLU6 primer set, the results obtained with this primer set were considered to be more consistent with microscopy.

DISCUSSION

Reliable, rapid, and inexpensive diagnostic methods are very important for appropriate management and treatment of malaria. Microscopic examination and rapid diagnostic tests are two main diagnostic tools (11). Microscopic examination is still gold standard depending on low cost, simplicity and reliability (11, 12). However, particularly in cases with low parasitemia, false negative results and risk of overlooking of one species in mixed infections with dominant species are its disadvantages (12–15). The reported minimum parasitemia density detectable with microscopic examination is 50 parasites/µl. In general, the reliability of microscopic results depends on experience and skills of microscopist. Similar morphology of certain *Plasmodium* species is one of the problem in diagnosis. Misdiagnosis of *Plasmodium* species, particularly overlooking of fatal *Plasmodium* species have a negative impact on prognosis of the disease, on efficacy of treatment and control measures (12, 16). As sensitivity of microscopic examination is low, rapid diagnostic tests or its combination with PCR assays is recommended for assessment of patients, who had previously malaria and suffered from relapse (17). Although rapid diagnostic tests have some advantages like getting results within half an hour and applicability by an unqualified technician, there are also some limitations like decrease of sensitivity in cases with a parasitemia density less than 100 parasites/µl, false positivity due to persistence of antibodies in circulation particularly after treatment and inability to differentiate *Plasmodium* species other than *P. falciparum* and *P. vivax* (4, 11, 12, 18, 19). The accurate diagnosis of the *Plasmodium* species is crucial for effective treatment of malaria. In recent years, several comparative studies focused on nested and multiplexed PCR methods or different primer sets were conducted in order to develop especially a molecular gold standard method or a reliable alternative molecular method (20). Although malaria is not endemic in our country, there is a considerable number of imported cases. As our laboratory is a national reference laboratory, a large number of blood samples are sent to our laboratory from 81 cities for verification. The majority of these samples have low parasitemia density or belong to patients, who had already received antimalarial treatment. In order to diagnose rapidly and reliably, the combined use of microscopic examination, rapid diagnostic tests, and molecular methods are preferred, as diagnostic sensitivity and specificity of each method are different.

Currently, molecular techniques are widely used in several studies and accepted as gold standard in laboratories depending on their very high sensitivity and specificity (21). PCR assay is a suitable technique for collection of epidemiological data and is a useful diagnostic tool particularly in regions with low incidence, in cases with low parasitemia and submicroscopic infections (17). Parasite densities ≥5 parasites/µl can be detected with PCR (4, 12). In recent studies, this limit was identified as 0.5 parasites/µl (12). The disease control and elimination of the parasite are very important in cases, in which parasitemia density is so low that it can be overlooked even with the microscopic examination (1). Nested PCR is generally accepted as molecular gold standard. However, it is time-consuming and labor-intensive method. As six separate PCR reaction is needed for determination of all species, a large number of reagens and consumables have to be used (12). On the other hand, cost of this technique may be decreased if it is used only for cases with high clinical suspicion and negative microscopic examination (11). Although there is a theoretical annealing temperature for primers, laboratory conditions like required reagens and other equipment may change. Therefore, the most suitable annealing temperature should be determined with a gradient study. At a low annealing temperature may proliferate non-specific products, but this problem can be eliminated later on with the application of higher annealing temperature. But this implementation decreases the amount of PCR product (22). $MgCl_2$ concentrations, which will be added to amplification mixture, is important for optimization of PCR assay. In our study, a gradient study for optimization of each primer pair and each PCR reaction was conducted with trial of several annealing temperatures, $MgCl_2$ concentrations, mixture densities and amplification conditions, which provide the best band images, were determined.

The targets used in molecular malaria studies involves 18S ssu rRNA, circumsporozoite surface protein, a nuclear gene encoding cysteine protease and some common components like genus-specific and species-specific sequences (12). The PCR-based diagnostic methods, which are targeting gene 18S rRNA, are used for determination of human *Plasmodium* species since 1990 s. Nested PCR using this gene is accepted as a standard method for molecular-based malaria diagnosis. Diagnostic sensitivity of these 18S rRNA based tests is between 1 and 10 parasites/µl. Along with nested PCR, antigenic genes such as mitochondrial cytochrome b, stevor and msa-2, mitochondrial regions PgMt19 and PfMT869, Pvr47 and Pfr364 genes, Pvr47 and Pfr364 genes enabled the screening of field samples in epidemiological studies and determination of submicroscopic malaria infections. However, sensitivity changes depending on used method and characteristics of targeted gene (23). In several studies, it was

reported that all subspecies were successfully detected with 18S rRNA based primers and their sensitivity and specificity were found to be higher than microscopic examination and rapid diagnostic tests (4, 11, 13, 14, 21, 24).

Anthony *et al.* accepted microscopic examination as gold standard and compared the sensitivity and specificity of two different primer sets targeting 18S rRNA and dhfr-ts for differentiation of *Plasmodium* species with nested PCR. According to their results, sensitivity and specificity of 18S rRNA based primers were 91.9% and 100%, respectively and the sensitivity and specificity of dhfr-ts based primers were 51.4% and 100%, respectively (24). Regarding research on nested PCR, there are several studies reporting that genus-specific rPLU1, rPLU5 based primers and species-specific based rFAL1, rFAL2, rVIV1, rVIV2, rOVA1, rOVA2 and rMAL1, rMAL2 primers had high sensitivity and specificity (5, 7, 15, 23–25). In addition, there is also a wide range of studies reporting that especially genus-specific rPLU5, rPLU6 based primers and species-specific based rFAL1, rFAL2, rVIV1, rVIV2, rOVA1, rOVA2 and rMAL1, rMAL2 primers had high sensitivity and specificity in the nested PCR (1, 6). In our study, we accepted microscopic examination as gold standard and blood samples, which were microscopically *P. vivax* positive, were processed with conventional PCR assay using the same primers and 98.21% of samples were positive with rPLU1, rPLU5 based primers and 97.02% were positive with rPLU5, rPLU6 based primers. In abovementioned study, which is consistent with our study, two primer sets could not be compared because of difference between sample matrixes. We did not find any other study focused on this topic.

It was reported that sensitivity of single-step PCR is significantly lower compared to nested PCR. In spite of this, determined detection limit was lower than microscopic examination (7). Depending on this information, it was suggested that rate of false negativity might be further decreased with use of nested PCR method and species-specific primers. In our studies, after first step PCR amplification with rPLU1, rPLU5 and rPLU5, rPLU6 based primer pairs, we obtained successful results with nested PCR methods and rVIV1, rVIV2, rFAL1, rFAL2, rMAL1, rMAL2 and rOVA1, rOVA2 based primers.

CONCLUSION

Microscopic examination is an essential method due to the low cost and acceptance as a gold standard. However, diagnosis of patients with low parasitemia density and mixed infection is critical for elimination of the disease. In addition, in laboratories with a heavy workload, where samples should be rapidly evaluated, microscopists practically cannot allocate 45–60 minutes for each blood sample. Therefore, these laboratories have to increase their diagnostic capacities with help of diagnostic methods, which have high sensitivity and specificity, relatively rapid and inexpensive, narrow diagnostic margins and require less experience.

In our study, although we observed no significant difference between two primer sets and determined a perfect consistency, it was decided to prefer rPLU1, rPLU5 primer pair in cases where routine PCR method should be used in our laboratory. Because this primer pair PCR amplification obtained more successful results. In this study, reason of false negativity obtained with both primer sets could not be explained because parasitemia levels of the samples determined positive by microscopy were not determined. This situation is considered to be a significant limitation of the study.

Compliance with Ethical Standards: This study was approved by the ethics committee of the Ankara Training and Research Hospital (No: 158/2019, Date: 16.01.2020).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - SU, BÇ; Design - SU, BÇ; Supervision - SU, BÇ; Analysis and/or Interpretation - SU, BÇ; Literature Search - SU; Writing Manuscript - SU; Critical Review - SU, BÇ

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study has received no financial support.

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