A simple, efficient and inexpensive method for malaria parasites' DNA catching from fixed Giemsa-stained blood slides

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Abstract As parasitological or microscopic method is the gold standard and the best method for diagnosis of malaria, so fixed Giemsa-stained blood slides in the form of thick and thin blood smears are the most important data collections of malaria, especially historical slides. The parasites are dead but their DNA is valuable for many molecular biologic researches. A simple and efficient method for catching and extraction malaria parasites' DNA with a desired yield from dried and stained blood on slides is the first and major step. Introduction of an applicable, efficient and inexpensive DNA catching method and assessment of its performance in following molecular applications was the main objective of present study.

Keywords Malaria · *Plasmodium falciparum* · *Plasmodium vivax* · DNA extraction · Fixed Giemsa-stained · Blood slide

Introduction

Malaria is a major tropical disease. It infects approximately 500 million people and causes 1.5–2.7 million deaths annually (World Health Organization, 1996. Fact Sheet No. 94. Geneva:World Health Organization). The tropical and sub-tropical regions are the most malaria endemic areas in the world (McMichael et al. 2012). The major prevalent species

causing malaria is *Plasmodium vivax* (*P. vivax*), in many malaria regions as well as Iran (Eskandarian et al. 2003).

The microscopic examination of Giemsa-stained thick and thin blood smears is the gold standard method for detecting plasmodium infections. Its sensitivity reaches to 50 malaria parasites in one microliter of blood (0.001 % of parasitemia) and accuracy of 98 % in determining the human plasmodium species. Although this method is effective and inexpensive, but it is time-consuming and requiring skilled microscopists. Moreover, its sensitivity drops in low parasitemia dramatically (Brown et al. 1992; Gilles 1993; Snounou et al. 1993).

Some other methods are used for detecting and identifying of malaria parasites such as polymerase chain reaction (PCR) which considerably increases the sensitivity and specificity. These methods have a promising future, especially in areas where two or more human plasmodium species are present simultaneously (Imwong et al. 2003). PCR technique is based on finding of a specific DNA. Such method successfully detects parasites in mixed and or low parasitemia infections. In comparison with microscopy, it has shown more sensitivity (Carrasquilla et al. 2000), validity and usefulness in epidemiological studies of malaria.

The positive slides of malaria which are in the form of thick and thin Giemsa-stained blood smears and remaining from past times are valuable collections of data from past history of malaria in a region that attract attentions. So ability to extraction and catching malaria parasites' DNA has a considerable importance for a variety of molecular researches in malaria.

The objective of the present study was to develop of a simple, rapid, inexpensive and sensitive method for extraction and purification of malaria parasites' DNA from positive blood smears for all forms of downstream molecular experiments.

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Materials and methods

Sample collection

This study was carried out on 50 fixed Giemsa-stained slides which have been recognized positive for *P. vivax* by microscopic method. They were taken from patients from malarious regions of south and south eastern Iran and transferred to our laboratory by post, during 2010–2012. Fifty microscopic confirmed *P. vivax* positive blood smears from 26, 17 and 7 patients from Sistan va Baluchestan, Hormozgan and Isfahan provinces, respectively were processed. From mentioned three provinces, the first two has recognized as endemic and the third non endemic regions for malaria in Iran.

After DNA extraction, and before running of PCR, the spectrophotometric characters of all samples were analyzed using NanoDrope spectrophotometer (WPA, Biowave UK) for measuring of extracted DNA quality and quantity.

DNA extraction

Snounou method (Snounou 1996) was used for DNA extraction from positive Geimsa-stained blood smears with some modification. Briefly, washing was done on all the surface of blood smear with ether and air dried. A drop of sterile PBS buffer (pH 7.4) was added to each slide and scraped the whole blood with sterile scalpel blade then the scraped material transferred into the 1.5 ml Ependorff tube contained 20 µl of 100 mM Na₂Hpo₄, and centrifuged at 26,000×g for 20 min. Supernatants were discarded, and 20 µl of 100 mM Na₂Hpo₄ were added to sediments. The washing step repeated for three times. Then after the supernatants were discarded, 10 µl of 5 mM Na₂Hpo₄ were added to the sediments and centrifuged at 26,000×g for 5 min. The above mentioned steps repeated for two times subsequently discarded supernatant, and dissolved sediment in 10 µl of sterile water, boiled for 10 min and then centrifuged at 10,000×g for 10 min, the supernatants were containing DNA. All extracted DNA samples analyzed for detailed characters using a WPA, Biowave UK NanoDrop spectrophotometer. 5 µl of extracted DNAs were used for PCR assay.

Nested PCR was used for demonstration of procedure efficiency. The *P. vivax* Dihydrofolate reductase (DHFR) gene was selected for amplification.

Primers

Two pair of primers as VDTOF and VDTOR were used for first round and VDNF57 and VDFNR for second round of nested PCR has obtained from Imwong et al. report (2003) (Imwong et al. 2003). The primers and their profile have shown in Table 1. All amplifications were done in a final volume of 25 µl.

Nested 1

Each 25 µl reaction contained: 5 µl template DNA, 250 nM of each primer, 10 mM tris Hcl (pH: 8.3), 50 mM KCl, 2 mM MgCl₂, 125 µM deoxynucleotid triphosphate, and 0.2U Taq polymerase (Invitrogen).

Nested 2

Each 25 µl reaction contained: 0.25 µl of round 1 PCR product as template DNA. The concentration of other combinations was similar to nested 1.

PCR condition

PCR running was carried out in 30 cycles for each nested round. In the first round thermal cycler condition was; 95 °C for 5 min as starting denaturation followed by 30 cycles of 95 °C for 1 min, 64 °C for 2 min, 72 °C for 2 min and 72 °C for 15 min as final extension. The second round amplification was similar to above condition except the annealing temperature that was 64 °C for nested 2.

The second round PCR product was electrophoresed on 1.5 % agarose gel containing green DNA viewer[®] and analyzed under UV eliminator in a gel document chamber.

Results

The amount of, OD260/OD280 and total double strand DNA for all samples were used as good marker for DNA quantity and quality and the yield efficacy of this method for extraction and purification of DNA and their PCR results compared with parasitemia (data not show). The results are summarized as following table (Table 2).

Table 1 Primers and profiles used for amplification of DHFR gene of *P. vivax*

Nested PCR	Primers	Sequence	Product size (bp)	References
Nest-1	VDTOF	ATGGAGGACCTTTCAGATGTATTTGACATT	1,869	Imwong et al. (2003)
	VDTOR	GGCGGCCATCTCCATGGTTATTTTATCGTG		
Nest-2	VDNF57	CATGGAAATGCAACTCCGTCGATATGATGT	472	Imwong et al. (2003)
	VDFNR	TCACACGGGTAGGCGCCGTTGATCCTCGTG		

Table 2 Characters of extracted DNAs from 50 malaria fixe Giemsa-stained blood smears using NanoDrop spectrophotometer

	OD280 (abs)	OD260 (abs)	OD230 (abs)	OD260/OD280	OD260/OD230	DsDNA (µg/ml)
1	2.17	346	4.88	1.60	0.71	173.1
2	2.63	4.18	5.63	1.59	0.74	209.029
3	9.88	11.93	23.36	1.21	0.51	596.9
4	4.33	4.73	7.37	1.09	0.64	236.4
5	6.45	6.97	15.66	1.08	0.45	348.99
6	3.29	3.61	8.39	1.10	0.43	180.9
7	3.46	3.79	9.23	1.10	0.41	189.9
8	3.46	3.79	9.23	1.10	0.41	189.9
9	3.65	4.32	7.09	1.18	0.61	216.2
10	1.85	3.10	5.96	1.67	0.52	155.09
11	2.78	3.58	6.49	1.29	0.55	179.1
12	2.09	2.30	6.15	1.10	0.37	115.07
13	1.45	3.94	1.56	1.07	0.40	78.2
14	1.51	1.58	3.79	1.05	0.42	79.48
15	2.89	3.25	7.57	1.12	0.43	162.5
16	9.96	10.56	22.5	1.06	0.47	528.5
17	5.70	6.023	14.19	1.06	0.43	301.7
18	6.23	6.90	12.17	1.11	0.57	345.3
19	5.12	5.78	10.90	1.13	0.53	289.2
20	2.45	2.65	5.38	1.08	0.49	132.8
21	10.3	11.24	26.53	1.08	0.42	562.02
22	2.42	2.76	6.28	1.14	0.44	138.23
23	1.16	1.22	2.64	1.05	0.46	61.14
24	1.02	1.058	2.51	1.03	0.42	52.92
25	1.95	1.87	3.78	0.96	0.50	93.87
26	1.17	1.18	2.41	1.01	0.49	59.44
27	1.40	1.72	3.04	1.23	0.57	86.11
28	2.79	3.40	6.59	1.22	0.52	170.10
29	1.11	1.32	2.76	1.19	0.48	66.28
30	1.46	1.93	3.50	1.32	0.55	96.95
31	0.95	1.00	2.12	1.05	0.47	50.16
32	8.11	9.45	13.05	1.16	0.72	472.7
33	1.30	1.50	3.00	1.15	0.50	75.31
34	1.10	1.13	2.53	1.03	0.45	56.71
35	1.78	1.92	3.87	1.08	0.50	96.12
36	4.15	4.67	10.62	1.12	0.44	233.53
37	1.33	1.39	3.86	1.05	0.36	69.63
38	8.57	9.50	13.75	1.11	0.69	475.1
39	6.01	6.15	18.74	1.02	0.33	307.5
40	0.670	0.663	1.990	0.972	0.156	0.160
41	0.803	0.774	2.075	0.901	0.168	0.171
42	0.721	0.821	2.203	0.996	0.171	0.186
43	0.618	0.719	1.168	1.409	0.437	0.226
44	0.112	0.118	0.187	1.088	0.517	0.048
45	0.423	0.461	0.567	1.185	0.696	0.158
46	0.075	0.093	0.138	1.429	0.571	0.039
47	0.437	0.442	0.613	1.031	0.490	0.107
48	0.219	0.211	0.309	0.922	0.492	0.063

Table 2 continued

	OD280 (abs)	OD260 (abs)	OD230 (abs)	OD260/OD280	OD260/OD230	DsDNA (µg/ml)
49	0.728	0.777	1.632	1.162	0.292	0.229
50	1.441	1.495	2.387	1.079	0.453	0.481

Fig. 1 Typical electrophorogram of nested PCR product. Electrophorogram representation of agarose gel electrophoresis of nested PCR on 1.5 % agarose gel for *P. vivax*. A 100 bp ladder DNA size marker, B negative control, C negative PCR, D positive control, E positive PCR (472 bp)

Overall, 2(4 %) sample had a ratio ≥ 1.5 , 5(10 %) < 1 and 43(86 %) between 1 and 1.5. The total DsDNA (µg/ml) had a wide range between 0.48 up to 596.9 µg/ml.

All of 50 samples revealed positive results in nested PCR. The electrophorogram of one sample has shown typically in Fig. 1.

Discussion

Although the parasitological or microscopic method is the method of choice or golden standard for diagnosis of malaria, there are some circumstances that require to additional diagnostic methods (World Health Organization, 1996). Investigation of several aspects of historical malaria has to do upon historical fixed Giemsa-stained blood slides. Diagnosis confirmation of very low parasitemia samples,

differentiation of reinfection from recrudescence or relapse, the genetic basis of drug resistance and classification and determination of clones and lineages of Plasmodia, are some cases of these conditions (Ebrahimzadeh et al. 2006; Eskandarian et al. 2003; Imwong et al. 2003; Scopel et al. 2004; Sultan et al. 2009). PCR technique has been the most effective one for many applications. It has shown high sensitivity and specificity in several experiments (Brown et al. 1992; Snounou et al. 1993). The diagnosis of malaria is a careful and difficult in the low parasitemia infections; on the other hand observing only one parasite is adequate for a correct detection. As a matter of fact the probability of false negative rises when the blood sample is from a low parasitemia patient (Edoh et al. 1997; Payne 1988). Moreover the microscopic method cut off is about 50 parasites in a microliter of blood. The PCR technique is more sensitive and its cut off is about 5 parasites/µl of blood, but the PCR requires sufficient template DNA (John et al. 1998). At the result, when somebody has to work on a historical blood slide, the efficiency of DNA extraction method is very important. A good method for DNA extraction and purification should be simple efficient applicable and inexpensive. There are lots of inventive method for DNA extraction from fixed Giemsa-stained slides with different efficiencies and applications. We used nested PCR for raising the sensitivity of PCR. Our results were coincidence mostly with others, but it is probably the first one which was done on the DNA amount and its quality and quantity specifically, so as our searches the papers from Iran and other countries showed, there isn't any especial report on this subject (Ebrahimzadeh et al. 2006; Imwong et al. 2003; John et al. 1998; Scopel et al. 2004; Sultan et al. 2009; Zakeri et al. 2002).

Conclusion

Using a well setting up DNA extraction method and PCR technique is the most efficient way to study the historical stained blood smears for different aspects of malaria.

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