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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Document heading doi: 10.1016/S2221-1691(15)30377-4

Limited genetic diversity among *Plasmodium falciparum* isolates using nested PCR in Jazan Area, Saudi Arabia

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PEER REVIEW

Peer reviewer

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Comments

This paper reports a primary result of genetic diversity of *P. falciparum* in Jazan Area of Saudi Arabia. The findings are important to those closely related research interests.

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ABSTRACT

Objective: To establish molecular characterization of *Plasmodium falciparum* field isolates in Jazan Area of Saudi Arabia measured with highly polymorphic genetic marker, *i.e.* the merozoite surface protein 2 (MSP 2).

Methods: Blood samples were collected from 128 clinically suspected patients attending both Jazan and Sabia hospitals, Kingdom of Saudi Arabia. Both hospitals reflected urban and rural settings respectively. Analysis of central polymorphic region of MSP 2 (3D7 and FC27 allelic families) was performed using nested PCR for malaria patients.

Results: For MSP 2 allelic families of *Plasmodium falciparum*, 16 cases (53.3%) carried FC27 type and 14 cases (46.7%) carried 3D7 type, whereas no malaria cases harbored both allelic types. The present study showed that in urban area, 80% of FC27 fragments were 500 bp while in rural area it was 45.5% ($P = 0.08$). The FC27 400 bp allele was more prevalent in patients from rural than those from the urban area ($P = 0.08$). The most prevalent infecting 3D7 allele was the 3D7 300 bp in both areas. In the present study, there were no multiple infections.

Conclusions: The limited genetic diversity which was observed in Jazan (considered as an endemic area) may be attributed to the small sample size or sustained malaria control program.

KEYWORDS

Plasmodium falciparum, PCR, Merozoite surface protein 2

1. Introduction

Malaria is among the most important of six diseases on the World Health Organization/Tropical Disease Research list[1]. Globally, an estimated 3.2 billion people are at risk of being infected with malaria and developing disease, and 1.2 billion are at high risk (> 1 in 1 000 chance of getting malaria in a year)[1]. According to the latest estimates, 198 million cases of malaria occurred globally in 2013 and the disease led to 584 000 deaths. The burden is heaviest in the World Health Organization African Region, where an estimated 90% of all malaria deaths occur, and in children aged under 5 years who account for 78% of all deaths[2].

Plasmodium falciparum (*P. falciparum*) is an important public health problem in Kingdom of Saudi Arabia (KSA)[3,4]. Jazan Region is considered as an endemic area, and malaria prevalence is higher in this area than in any other regions of KSA[5]. Jazan Area consists of many villages scattered with less advanced network of roads when compared with other regions of KSA. There are many factors that increase number of mosquitoes throughout the year as water reservoirs. Malaria health records indicated that the discovered malaria cases were 1 236 per 100 000 inhabitants in 1992, but dropped to 276 cases in 2000[6]. Malignant malaria parasite (*P. falciparum*) is responsible for 90% of cases in Jazan. The main vector of the disease is *Anopheles* species[5,7].

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Article history:
Received 27 Oct 2014
Received in revised form 5 Nov 2014, 2nd revised form 26 Feb 2015
Accepted 28 Feb 2015
Available online 27 Mar 2015

Population genetic analyses of *P. falciparum* in Southeast Asia have been reported from Thailand, Malaysia, Myanmar, Philippines, Iran and Pakistan[8-13]. The genetic diversity among the *P. falciparum* population is an important indicator of the intensity of malaria transmission in an area[14]. A high endemic area showed generally numerous genetic diversity and infected cases carrying different genotypes. In contrary, studies of parasite population in a low endemic area showed a limited genetic diversity and the majority of infections are monoclonal[14].

Introduction of PCR genotyping in malaria research improved the understanding of parasite biology[15]. A number of highly diverse genetic markers of *P. falciparum* have been identified and studied as potential vaccine candidates[16,17]. The most common markers used for analyzed genotyping of *P. falciparum* are the surface antigens merozoite surface protein 1 (MSP 1), MSP 2 and the glutamate-rich protein[18]. Three genetic markers are different, *i.e.* located on different chromosomes, single copy genes with extensive polymorphism regarding to sequence and size[19]. These features make them suitable candidates for researches where identification and enumeration of genetic distinct *P. falciparum* parasite sub-populations are of interest. They have been proven to be useful tools in both molecular epidemiology studies as well as determination of treatment failures from new infections in antimalarial drug trials[18,20]. *P. falciparum* field isolates have been characterized in different area such as Iran, India and Pakistan but not previously in Jazan Area, KSA, using MSP 2 surface antigen. This study, therefore, aimed to measure molecular characterization of *P. falciparum* isolates in Jazan Area, KSA using highly polymorphic genetic marker, MSP 2.

2. Materials and methods

2.1. Sample collection

The study was performed from January 2013 to August 2014. Blood samples were collected from 128 suspected patients during a three-month period from January to March. Parasitological work of the present study was done in Faculty of Pharmacy, Jazan University KSA while molecular work was done in Biotechnology Center, Cairo University. Patients aged between 6 and 45 years old with fever or a history of fever within the past 48 h. No history of antimalarial drug administrated in the last two weeks. There was no evidence of a concomitant febrile disease. Blood samples were collected from 128 clinically suspected patients attending both Jazan and Sabia hospitals, KSA. Both hospitals reflected urban and rural settings respectively. Patients were diagnosed to be infected with *P. falciparum* using ICT optimal test (ICT-Amrad, Sydney, Australia) then it was confirmed by thin blood film stained using Giemsa stain (Sigma-Aldrich) to detect asexual stages of the parasites. Blood films would have been considered negative if no parasites were seen in 300 oil immersion fields on thin blood film.

2.2. DNA extraction

DNA was extracted using the GF-1 tissue DNA extraction kit from whole blood and finally suspended in 30 μL of double-distilled sterile water[21]. The template was kept frozen until used for analysis.

2.3. PCR amplication

PCR amplification of template DNA and analysis of central polymorphic region of MSP 2 (3D7 and FC27 allelic families) were performed according to the recently recommended genotyping protocol with minor modifications[22].

A polymorphic locus, MSP 2, was used for the genotyping of the parasite population in the present study. The regions of MSP 2, which vary in repeat number and in sequence type, with two (FC27, 3D7) allelic families, were analyzed by a nested PCR amplification[23].

Primary amplification reactions were carried out using 5 μL of DNA extracted solution and the nested PCR used 2 μL of the first PCR product. The following oligonucleotide primers sequences were used[24]: MSP 2-1 outer (ATGAAGGTAATTAAAACATTGTCT ATTATA); MSP 2-4 outer (ATATGGCAAAAGATAAAACAAGTGTTG CTG); FC27-A (GCAAATGAAGGTTCTAATACTAATAG); FC27-B (GCTTTGGGTCCTTCTTCAGTTGATTC); 3D7-A (GCAGAAAGT AAGCCTTCTACTGGTGCT); 3D7-B (GATTTGTTTCGGCATTAT TATGA). For each reaction, 200 μmol/L deoxy-ribonucleoside triphosphate, 1 μmol/L primer, 0.5 IU of Taq DNA polymerase (Sigma Aldrich Chemise GmbH, Deutschland) and PCR buffer pH 8.3 at 25 ℃ containing 100 mmol/L Tris-HCl, 500 mmol/L KCl, 15 mmol/L $MgCl_2$ and 0.01% gelatin were used to prepare a volume of 20 μL. First and nested amplifications were performed on a peltier thermal cycler-100. The cycle condition for MSP 2 outer PCR (30 cycles) consisted of denaturation at 94 ℃ for 5 min, annealing at 55 ℃ for 1 min 30 seconds, extension at 72 ℃ for 2 min and final extension for 10 min to insure that all products were full-length. For the inner PCR similar conditions were used for the denaturing and extension but the annealing conditions were specific for each primer as described: FC27 and 3D7 were annealed at 57 ℃[24]. The specific amplifications in MSP 2 group, the reaction were 30 cycles. Precautions were carried out to optimize and standardize the PCR conditions including DNA extraction methods, template volumes, Mg^{2+} concentrations and annealing temperature. The amplified PCR products were analyzed using agarose gel (1.5%) electrophoresis (Sigma Aldrich Chemise GmbH, Deutschland). The sizes of the PCR products were estimated using 100 bp DNA ladder marker (Sigma Aldrich Chemise GmbH). The MSP 2 alleles were characterized by their molecular weights and considered the same if their molecular weights were approximately within 10 bp[24]. A single PCR fragment detection of this locus was classified as an infection with one parasite genotype. The detection of more than one PCR fragment for MSP 2 locus (*i.e.* an infection with more than one parasite genotype) is known as a multiple *P. falciparum* infection.

2.4. Statistical analysis

The DNA fragments from the electrophoresis were assigned to specific allelic families according to the second PCR results. The data were double entered and were analyzed using SPSS V. 16. The proportions comparison was made by *t*-test and Fisher's exact test. Significance levels were at $P < 0.05$.

2.5. Ethical considerations

Blood samples were collected following informed consent from patients. All patients were treated and followed by clinician.

3. Results

Blood samples of 128 suspected cases were collected from two different localities in Jazan Area. Among them, 30 cases were infected by *P. falciparum* (23.4%). About 17/128 (13.3%) malaria patients were from Sabia hospital and 13/128 (10.2%) were from Jazan hospital (Table 1). It was found that 76.7% of positive blood samples belonged to Saudi citizens. For MSP 2 allelic families of *P. falciparum*, 16/30 malaria cases (53.3%) carried FC27 type and 14/30 malaria cases (46.7%) carried 3D7 type, whereas no malaria cases harbored both allelic types (Table 2). For the MSP 2 alleles, prevalence of the 3D7 type allele and the FC27 allele among urban and rural areas of Jazan were shown in (Table 2). The MSP 2 alleles were classified according to their size. Seven individual MSP 2 alleles were identified in study patients in the two sites (Figure 1): 4 different alleles for FC27 (300-400-450-500 bp) and 3 alleles for 3D7 (250-300-350 bp). Fragment sizes of all the FC27 type alleles from patients living in the urban area were between 300 bp and 500 bp (Figure 1). The present study showed that in urban area, 80% of FC27 fragments were 500 bp while in rural area it was 45.5%. (P = 0.08). The FC27 400 bp allele was more prevalent in patients from rural than those from the urban area (P = 0.08) (Figure 2). The most prevalent infecting 3D7 allele was the 3D7 300 bp in both areas (50% in urban and 83.4% in rural area) (Figure 1). In the present study there were no multiple infections (Figure 2).

Table 1

Sex and nationality of the patients infected by *P. falciparum* [*n* (%)].

District	Sex		Nationality		Infected patients in total samples
	Male	Female	Saudi	None Saudi	
Jazan	10 (76.9)	3 (23.1)	9 (69.2)	4 (30.8)	13 (10.2)
Sabia	12 (70.6)	5 (29.4)	14 (82.4)	3 (17.6)	17 (13.3)
Total	22 (73.3)	8 (26.7)	23 (76.7)	7 (23.3)	30 (23.4)

There is no significance among the data.

Table 2

DNA amplification success rate for each marker gene in blood samples from two different localities in Jazan Area.

Area setting	PCR positive samples [*n* (%)]		Total
	FC27 MSP 2 alleles	3D7 MSP 2 alleles	
Urban (Jazan)	5 (38.5)	8 (61.5)	13
Rural (Sabia)	11 (64.7)	6 (35.3)	17
Total	16 (53.3)	14 (46.7)	30

There is no significance between the data.

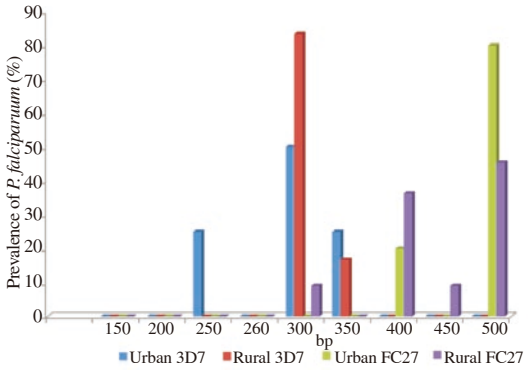

Figure 1. Prevalence of *P. falciparum* 3D7 and FC27 MSP 2 alleles from urban and rural area in Jazan classified by bp.

P value is significant (P < 0.05) in FC27 allele and not significant in 3D7 allele using *t*-test.

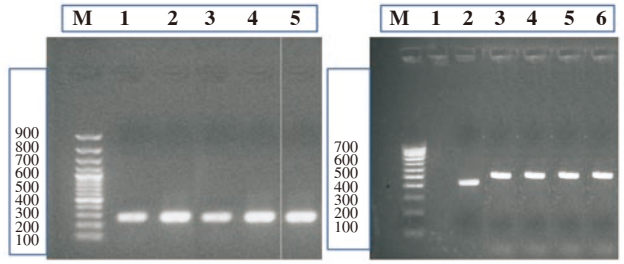

Figure 2. PCR genotyping of polymorphic alleles of MSP 2. a: 3D7 allele, band size (300 bp); b: FC27 allele, band size (Lane 2: 400 bp), (Lanes 3, 4, 5, 6: 500 bp).

M: DNA marker.

4. Discussion

Malaria is endemic in Saudi Arabia. Although it is significantly controlled by Ministry of Health, patients with clinical symptoms of malaria are continuously discovered[25]. In the present study, it was found that prevalence of *P. falciparum* infection was 23.4%. This result is in accordance with study done by Al-Jaser[6]. The majority of patients (23, 76.7%) were Saudi. Most of the patients (22, 73.3%) were males. These results were in agreement with a recent report from the same area by Bin Dajem *et al*[26].

The present study showed that FC27 alleles were predominant among malaria positive cases. Accordingly, many researchers reported the same results in Central Sudan and Eastern Sudan[27-29]. In contrary, Ghanchi *et al*.,[30] found that 3D7/IC alleles were more predominant than FC27 alleles in Pakistan while in sub-Saharan Africa, total of 116 different MSP 2 genotypes were recorded at the 5 different study sites, of which, 71.6% and 28.4% belonged to the 3D7 and FC27 allelic families, respectively[18]. These different results may be due to different geographical region. The present study showed that no cases harbored both alleles. This is in accordance with a study done by Bin Dajem *et al*.[26]; it was found that the vast majority of isolates (95%) carried a single clonal infection with a single allele at each of the examined genes (MSP 1 and MSP 2) in Jazan Area, Saudi Arabia. There were no multiple infections in present study. This result was dissimilar to many researchers done in Eastern Sudan[27], Uganda[31] and in Burkina Faso[24] where in the latter, frequency of multiple infections was > 90% in all infected children. In a high endemic area, there was generally extensive genetic diversity and infected cases almost carried different genotypes[14]. The low genetic diversity of the *P. falciparum* in Jazan Area probably reflects the impact of sustained control measures, where limited transmission can restrict the gene pool[28]. This low genetic diversity may be attributed to small sample size in the present study. Thus, low level of diversity seen in Jazan is rare and seen typically in limited endemic sites such as island populations in Papua New Guinea and the Solomon Islands[32].

The results revealed a relatively limited genetic diversity and no multiple infections. Differences in transmission level and antimalarial immunity were known to be strain specific[33]. This

may explain the differences in the distribution of the different alleles[34]. It was known that there was an increase of the migration process from the rural area to the urban area in the past ten years. This demographic change in the urban area, as well as individual and household factors (*e.g.* impregnated bed nets, insecticide spraying), and climatic and topographical factors (*e.g.* rain fall, temperature, humidity, the presence of water reservoirs) may affect the level of malaria transmission and lead to a rural-urban difference in parasite genotypes carriage. In contrary to our study, Ghanchi *et al*., found that for MSP 2 allelic families, 44% of the infections carried FC27 type and 40% carried 3D7/IC type, whereas 16% of the infections harbored both allelic types in Pakistan[30].

In the present study, two allelic types of MSP 2 were equally distributed with proportion of FC27 and 3D7 ranging between 38.5%-64.7% and 61.5%-35.3%, respectively, in isolates from urban and rural area. In a given locality, the parasite genetic pool might be relatively stable, perhaps due to the stable parasite life cycle. Thus, the distribution of alleles may be determined randomly so that certain alleles will predominate accidentally. However, there are certain factors affecting allelic distribution addition to the natural life cycle as antimalarial drug pressure which is characteristically greater in urban areas than rural one and the proximity of populations to water reservoirs as it has been found in Jazan. This results in a higher prevalence of drug resistance markers[35]. The use of insecticide treated nets or certain human genetic factors (hemoglobin types, glucose-6-phosphate dehydrogenase), that might explain the difference in the distribution of *P. falciparum* genotypes between the two sites.

The present work demonstrated that there were some differences in the *P. falciparum* diversity between patients living in urban and rural areas and this should be taken into account when designing MSP 2 malaria vaccine. The study also emphasizes the importance of evaluating the extent of parasite genetic variation and the factors affecting this variation. In this aspect, longitudinal studies to examine the dynamics of the *P. falciparum* genetic diversity, including genes conferring drug resistance, between urban and rural areas could play an effective role in malaria control.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Many thanks to doctors and technicians in duty of Parasitology Labs of Sabia and Jazan hospitals for their help for providing us with all parasitological data of patients.

Comments

Background

The genetic diversity of *P. falciparum* is important for its biology, transmission and treatment in any geographical areas. In Saudi Arabia, researchers of malaria including genetic diversity of parasites are rare in literatures.

Research frontiers

The research primarily identified the genetic diversity of *P. falciparum* in Jazan Area of Saudi Arabia.

Related reports

The genetic diversity of *P. falciparum* from the main malaria endemic areas of the world has been reported. MSP 1, MSP 2 and glutamate-rich protein are commonly used to type gene and its mutation of *P. falciparum*.

Innovations and breakthroughs

The research used MSP 2 to do genotyping of *P. falciparum*. However, more and more researchers used glutamate-rich protein to identify gene types and its mutation.

Applications

This is a primary identification of genetic diversity of *P. falciparum* in Jazan Area of Saudi Arabia.

Peer review

This paper reports a primary result of genetic diversity of *P. falciparum* in Jazan Area of Saudi Arabia. The findings are important to those closely related research interests.

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