Original Article

Molecular Characterization of Genetic Diversity of *Plasmodium falciparum* Associated with Imported Cases of Malaria in Southeastern Region of Saudi Arabia

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ABSTRACT

Growing global international travel has reflected significantly on the number of imported cases of malaria into non endemic regions of the world inclusive of Saudi Arabia were transmission of the disease is significantly controlled. This success in control could be challenged by the influx of imported cases of the disease. This research looks at the genetic diversity in merozoite proteins 1 and 2 (*msp* 1 and *msp* 2) of *Plasmodium falciparum* gene in imported malaria cases. Blood samples collected by pathology department were preserved as dried blood spot on WhatmanTM 903TM Protein saver card. Extraction of genomic DNA was with Qiagen QIAamp DNA kit Using primary and secondary nested PCR, *Plasmodium* species were classified with targeted primers. The nucleotides of KI, MAD_{20}, RO33 msp 1 and 3D7, FC27 *msp* 2 of *P. falciparum* gene were sequenced by Macrogen (South Korea) and analyzed with BioEdit version 7 software. Frequencies of distribution of *msp* 1 and *msp* 2 allelic families varied, with statistically significant differences (*p*-value 0.001). Mono and poly clonal infections were also encountered. Clonal fragment sizes ranged between low (110 bp) to high (625 bp). Sequence analysis showed genetic diversity in imported malaria cases in the present study, indicating that current control measures might not be adequate to maintain the status quo.

Key words: Malaria, Imported, *Plasmodium falciparum,* Merozoite proteins, *msp* 1, *msp* 2, Genetic, Diversity.

INTRODUCTION

Malaria is described as the "undisputed queen of parasitic diseases",[1] and this was attributed to a number of reasons. At the eighth World health congress in 1955, malaria was the first human parasitic disease scheduled for global eradication.[2] Today, the disease remains one of the most important parasitic diseases of man that has led to more deaths than those of all other combined parasitic diseases.[2] The World malaria[3] report placed estimated global cases at 228 million in 2018 with about 405,000 recorded deaths. In the same year, the World malaria report indicated that 61 indigenous cases of malaria were reported in Saudi Arabia while there were 2517 other reported cases of imported malaria. This shows the vulnerability of the Kingdom to imported cases malaria. For malaria, travel remains a risk factor with a recent literature[4] citing that the increase in cases of malaria could be attributed to continuous long distance travels as well as the huge global migration movement. All these factors, are components that could be a lead in characterizing the epidemiology of imported malaria in non-endemic countries.[4] Cross border importation between neighboring

Submission Date: 09-07-2021;
Revision Date: 09-09-2021;
Accepted Date: 15-10-2021.

DOI: 10.5530/ijper.55.4.218

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countries and Saudi Arabia remains of great public health importance[5] with sources of imported malaria grouped into three main types.[6] The first of these sources are those of expatriate work force, while others include yearly visits from all geographical regions of the world for religious rites and national borders with disease endemic countries such as the national border with the republic of Yemen. All these sources constitute a public health problem in the control of malaria a view shared by other researchers.[7,8]

Generally, the topography of Saudi Arabia is divided into groups based on incidences of transmitted cases of malaria. There is the non-malarious based, made up of the central, the north and eastern regions of the Kingdom that have terminated the transmission of the disease through effective vector control measures. There is also the low transmission areas in the Western parts while the high/medium transmission zones are in the southern and south-western parts of the Kingdom.[9]

Thus, these regions in Saudi Arabia could be grouped from high risk (Jeddah, Mecca, Taif, Najran), to low risk or no risk. Dammam, Dhahran and Hofuf the region of the present study, are in the low or no risk region. However, due to the high yearly migration into the Kingdom, there will be the need for close monitoring. On the other hand, there are mosquitoes in these localities and they could probably, be potential vectors in the region. Subsequently, imported cases of malaria in the Eastern Province of Saudi Arabia should be of a major concern and be under continuous monitoring. Generally, molecular epidemiological studies of malaria can be used to study the genetic diversity of infections in relation to disease phenotypes. Generally, the identification of parasite molecular markers involved in resistance to antimalarial compounds are known to be of great interest in the monitoring of development and spread of resistance. Also, individuals infected with *Plasmodium falciparum* often consist of genetically distinct parasite populations, such as clones of same parasite species.[10,11] Malaria parasite genotyping is an important tool in determining the multiplicity of infections [MOI] *of P. falciparum*.[12] It had been documented that *P. falciparum* infections in patients could present as multiple parasite genotype variants, which could play a role in parasite evolution.[13] However, the relationship between the numbers of *Plasmodium falciparum* genotypes, multiplicity of infection (MOI) continues to receive attention from researchers. Literature is silent on the genetic diversity of *Plasmodium* species associated with imported cases of malaria in this southeastern region of Saudi Arabia either for indigenous malaria infections or of visiting/migrant workers. The present investigation therefore aims at looking into the genetic diversity in imported cases of malaria in this south-eastern region of Saudi Arabia. It is expected that the obtained results will be important information for the effective management and in keeping the disease under control in the kingdom in general. This is in view of the fact that Saudi Arabia has a dynamic workforce migration.

MATERIALS AND METHODS

Study area and Ethical consideration

The study was carried out in the south-eastern region of Saudi Arabia. No patients were directly involved in the study. Samples were those that had tested positive to *Plasmodium* parasites and stored in the pathology department of the college of medicine. The samples had originated from visitors to the Kingdom and formed part of routine healthcare checks, which is a pre-requisite for employment. Samples were collected between from January 2018 to January 2020.

Molecular diagnosis of malaria parasites, collection and preparation of dried blood spot (DBS) samples

Conventional microscopic examination of thick and thin blood smears as recommended by WHO were used in the hospital laboratories to confirm the presence of malaria parasite. Dried blood spots (DBS) samples were prepared according to the standard operating procedures[14] on WhatmanTM 903TM Protein saver card [GE Healthcare UK] with 40–50 μL of blood introduced into each circle at the center point. One card was used per sample and given a code, date of sample collection, gender and age of patient. Collected blood spots were allowed to air-dry at room temperature and then kept in sealed labelled bags with silica desiccant and stored at -80°C until when they were examined. Earlier described protocol[15] was used for the amplification of 18S ribosomal RNA through primary and secondary nested PCR using specific primers probes for *P. falciparum, P. malariae and P. vivax* were used shown in Table 1.

Extraction of genomic DNA from DBS

Isolation of genomic DNA was carried out with Qiagen QIAamp 96 Blood DNA extraction kit following manufacturers' guidelines. With a paper puncher, 3mm in diameter punches were cut out from each DBS card and placed in 1.5 ml micro centrifuge tubes. To each tube was added 180 μL of ATL buffer and incubated at 85°C for 10 min, followed by the addition of 20 μL stock solution of proteinase K. The resultant mixture was mixed thoroughly by vortexing, incubated for 10 min at 70°C. To the resulting mixture 200 μL of 100% ethanol was added, mixed thoroughly by vortexing

Table 1: Primers used for nested PCR 18S ribosomal RNA amplification. PCR Primer Primer sequence (5'-3') PCR bp Plasmodium species

PCR	Name of Primer	Primer sequence (5'-3')	PCR bp	*Plasmodium* species
Primary	rPLUf	TTAAAATTGTTGCAGTTAAAACG	1.6 - 1.7	*Plasmodium genus*
	rPLUr	CCTGTTGTTGCCTTAAACTTC		
2nd Nested	rFALf	TTAAACTGGTTTGGGAAAACCAAATATATT	206	*P. falciparum*
	rFALr	ACACAATGAACTCAATCATGACTACCCGTC		
	rMALf	ATAACATAGTTGTACGTTAAGAATAACCGC	145	*P. malariae*
	rMALr	AAAATTCCCATGCATAAAAAATTATACAAA		
	rVIVf	CGCTTCTAGCTTAATCCACATAACTGATAC	121	*P. vivax*
	rVIVr	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA		
	rOVAf	ATCTAAGAATTTCACCTCTGACATCTG	226	*P. ovale*
	rOVAr	ATCTCTTTTGCTATTTTTTAGTATTGGAGA		

Table 2: Primers sequences used for genotyping by PCR for *msp* 1 and *msp* 2

Primer name	Round of PCR	Sequences (5'-3')	Allelic family	Annealing temp.	control	PCR Cycling parameter
msp 1	External Primary	CTAGAAGCTTTAGAAGATGCAGTATTG-F		61°C		Initial denaturation 5 minutes at 94 °C; PCR: Denaturation 94 °C for 1 min, annealing 61 °C for 45 secs; Extension 72 °C for 90 secs. {40 circles each}. Final extension 72 °C for 5 min. Hold 4°C.
		CTTAAATAGTATTCTAATTCAAGTGGATCA-R				
	Internal Secondary	AATGAAGAAGAAATTACTACAAAAGGTGC-F	K1	61°C	3D7	Initial denaturation 2 minutes at 94 °C; PCR: Denaturation 94 °C for 1 min, annealing 61 °C for 45 secs; Extension 72 °C for 90 secs. {30 circles each}. Final extension 72 °C for 10 min. Hold 4°C
		GCTTGCATCAGCTGGAGGGCTTGCACCAGA-R				
		AAATGAAGGAACAAGTGGAACAGCTGTTAC	AD20		HB3	
		ATCTGAAGGATTTGTACGTCTTGAATTACC				
		TAAAGGATGGAGCAAATACTCAAGTTGTTG-F	R033	63°C	R033	
		CATCTGAAGGATTTGCAGCACCTGGAGATC-R				
msp 2	External Primary	MSP2-1: ATGAAGGTAATTAAAACATTGTCTATTATA-F		55°C		Initial denaturation: 94 °C for 5 min; PCR: 40 cycles of 94 °C for 1.5 min, 55 °C for 45 s, 72 °C for 1.5 min; final elongation: 72 °C for 10 min. Hold 4°C
		MSP2-4: ATATGGCAAAAGATAAAACAAGTGTTGCTG-R				
	Internal Secondary	A1: GCAGAAAGTAAGCCTTCTACTGGTGCT-F	IC/3D7	55°C	3D7	Initial denaturation: 94 °C for 2 min; PCR: 30 cycles of 94 °C for 1.5 min, 55 °C for 45 s, 72 °C for 1.5 min; final elongation: 72 °C for 10 min. Hold 4°C
		3D7 A2: GATTTGTTTCGGCATTATTATGA-R				
		FC27 B1: GCAAATGAAGGTTCTAATACTAATAG-F	FC27		HB3	
		FC27: B2 GCTTTGGGTCCTTCTTCAGTTGATTC-R				

before centrifuging briefly. The complete process of parasite DNA extraction was carried out according to the detail protocol in the handbook of Qiagen-DNA purification from DBS.

Plasmodium parasite genotyping

Distinct subpopulations present in *P. falciparum* parasites are generally characterised by genotyping and this was carried out through nested PCR of extracted DNA. Thus, for their extensive polymorphism in size and sequences, genetic markers of merozoite surface protein (*msp*) 1 and 2 were chosen for the investigation. The allelic types for *msp* 1, K1, MAD_{20} and RO33, those of *msp* 2, FC27 and IC/3D7 were investigated with specific primers in the secondary nested PCR. A combination of methods described by Biodefence and emerging infections research resources repository (BEI) methods for Malaria research as well as the protocol for *msp* 2 genotyping by University of Maryland School of Medicine, malaria group[16] version were used for parasite genotyping. With the first amplification reaction, oligonucleotide primers shown in Table 2 were used for the first nested PCR. The resultant product from the first amplified reaction served as DNA template for the second nested PCR process using specific-sequenced primers for *msp* 1 and *msp* 2 as described previously.[15,17] The final volume of the primary and secondary PCR constituted products was 25 µL each. This was made up of the following, sterile water 17.4 µL; 2.5 µl of 10x buffer; 0.2 µL of dNTP (25mM); 0.8 µL of $MgCl_2$ (50mM); 0.5 µL each (10 µM) of forward and reverse primers; 0.2 µl of Tag polymerase and 3.0 template DNA. Thus, tubes targeting the allelic families of both *msp* 1 and *msp* 2 were set up. Bio-Rad thermal cycler was used for PCR amplifications with 40 and 30 cycles for primary and secondary reactions respective as shown in Table 2.

Detection of *msp* 1 and *msp* 2 alleles by Gel electrophoresis

The resultant secondary PCR product were analysed on 1% agarose gel by electrophoresis. 4 µL of amplified DNA products were stained with Ethidium bromide and documented with an electronic photographic documentation system. Amplicon sizes were detected as previously described[17] using a 100bp DNA ladder (Promega USA). DNA positive controls from BEI resources (www.beiresource.org) were used for the comparison of the DNA fragments of *Plasmodium* parasite species with molecular weights applied in characterising *msp* 1 and *msp* 2 alleles.

Determination of multiplicity of infection (MOI)

MOI or the complexity of infection for *msp* 1 and *msp* 2 was determined for positive samples according to set guidelines.[17,18] The number of genotypes detected per infection was determined by dividing the total number of alleles individually for *msp* 1 and *msp* 2 by the total number of samples that were positive to the markers of the genes. Isolates were grouped as either monoclonal or polyclonal infections if they carried a single allele or more than one genotype respectively.[19]

Sequencing of *msp* 1 *and msp* 2 *P. falciparum* gene and sequence analysis

DNA PCR products of KI, MAD_{20}, RO33 *msp* 1 alleles and 3D7 and FC27 *msp* 2 allele of *P. falciparum* were constituted with the specific primers and submitted to Macrogen (South Korea) in individual 1.5 µL micro-centrifuge tubes for PCR-clean up and bidirectional sequencing. Pre-sequence sample preparation was according to the Macrogen-sample guide. Resultant sequences were analysed using available online tools at National Center for Biotechnology information (NCBI) https://www.ncbi.nlm.nih.gov/nucleotide/. Also, BLAST (Basic local alignment search tool) https://blast.ncbi.nlm.nih.gov/Blast.cgi was used in finding regions of similarities in nucleotide/protein sequences as well as statistical analysis related to the sequences. Also, BioEdit version 7 was use for entropy Hx plot of amino acids sequence analysis.

Statistical analysis

GraphPad Prism version 8 was used for statistical analysis. Data is presented as percentages while the one sample t-test was used to compare statistical differences between monoclonal and polyclonal infections. Also, two sample t-test was used to compare differences in number of clones of *msp* 1 and *msp* 2 malaria infections with significant difference taken at $p<0.05$. Sequences analyses were carried out with BioEdit version 7. Also, entropy plots were obtained using BioEdit version 7 software. Phylogenetic trees for obtained sequences were inferred using the Neighbor-Joining method with all trees drawn to scale. Positions containing gaps and missing data were eliminated. While evolutionary analyses were conducted in MEGA5.

RESULTS

Demographic description of collected samples

Fifty-four (54) positive malaria samples were collected for over a period of two years (Figure 1A). Samples were

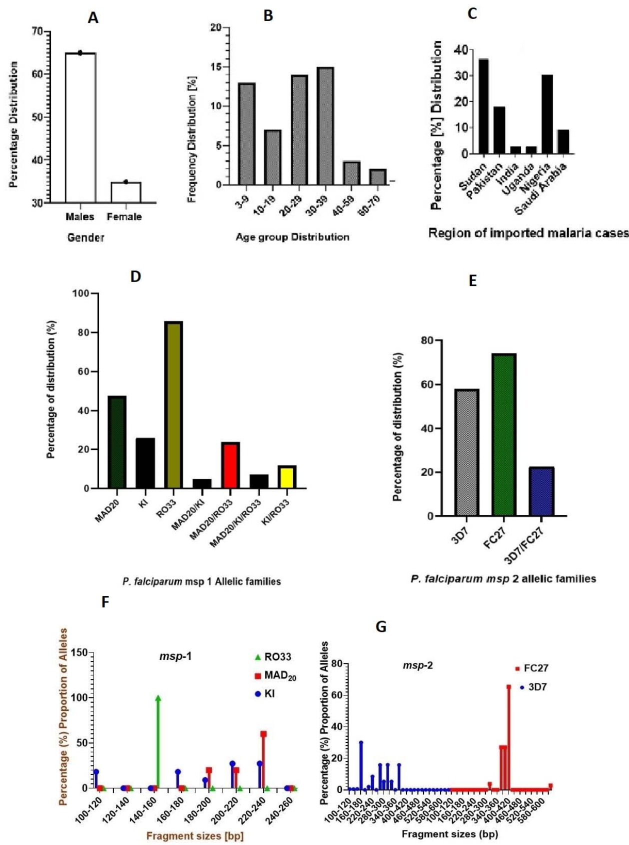


Figure 1: Showing demographic description (A-B), the geographical region of imported malaria cases (C), encountered allelic families of *msp* 1 and 2 Plasmodium falciparum gene (E-F) and Percentage distribution of fragment sizes for the allelic families KI, MAD20, RO33-msp 1 (G) and 3D7, FC27-*msp* 2 (H).

from males (65%) and females (35%). There was 9% of cases imported from the South-western part of the Kingdom, to the region of the study. The highest number of samples (35.2%) were from the east African country of Sudan. Others included travelers from the West African country of Nigeria (30%). While the remaining were from Pakistan (18.5%), India and Uganda, which constituted of 3% each of the total number of samples (Figure 1B).

Results showed age groups ranging from 3 to 70 years of age (Figure 1C). The highest samples were from the 30 – 39 age group. Others within age groups of 20-29 and 3 – 9 years age groups were more than of 40 years old and above.

Encountered species of Plasmodium parasite

Using nested PCR (nPCR), with specific primers targeting 18S rRNA gene, malaria parasite was encountered in 51 samples and were grouped species of *P. falciparum* (*n*47 = 92.2%) and *P. vivax* (*n*4 = 7.8%). Other *Plasmodium* species, *P. ovale* and *P. malariae* were not detected. In terms of species by country of importation, results showed *P. falciparum* to be the most and was encountered in samples from all the countries with the exception of India which had only *P. vivax*. Samples of *P. falciparum* parasite were genotyped by nested PCR for *msp* 1 and *msp* 2 for the investigation of molecular analysis of clonal distribution of the infections.

Table 3: Multiplicity of infection associated with *msp* 1 and *msp* 2 genes of *P. falciparum* isolates of imported malaria.

Gene	MOI	Monoclonal	Polyclonal	Trimorphic
		N (%)	N (%)	N (%)
Msp 1	[42/67] 1.6	22/42 = 52.38%	17/41 = 0.48%	3/42 = 7.14%
Msp 2	[41/31] 1.32	22/31 = 70.97%*	6/31 = 19.35% *	3/31 = 9.67%

MOI = Multiplicity of infection. * = significant $p< 0.05$

Encountered *msp* 1 *and msp* 2 allelic families of imported cases of malaria

PCR fragments amplification obtained were used in grouping the allelic families of *msp* 1 and *msp* 2. Results showed alleles for *msp* 1 to be more frequently encountered (82.35%) in the infections than those of *msp* 2 (60.8%). The allelic families of *msp* 1 included MAD_{20}, KI and RO33 all of which had been detected at varying degrees (Figure 1D) with RO33 being predominantly more as compared to MAD_{20} and KI. For *msp* 2, the detected allelic families were 3D7 and FC27 of the PCR amplified samples. In this group, the FC27 allelic family was predominant (74. 2% *n* =. 23/31) while 3D7 allelic family was the least (58.1% *n* = 18/31)

(Figure 1E). *P. falciparum* infections were grouped either as monoclonal or polyclonal. There were more monoclonal than polyclonal infections involving both allelic families of *msp 1* and *msp 2* (Table 3). A t-test comparison showed differences in percentages to be statistically significant with *p*-value of 0.001. In *msp* 1, more than half (52.38%) of the samples were monoclonal infections (Table 3). However, the differences between them and the polyclonal infections was not statistically significant (*p*-value = 0.74 at 41d*f*). Also, in the *msp 2*, alleles, there were more (70.97%) monoclonal than polyclonal infections (29.02%) with differences statistically significant (*p*-value = 0.015 at 30 d*f*) (Table 3). Trimorphic infections which was classified as samples that had 3 allelic families in *msp 1* alleles constituting of MAD_{20}/KI/RO33 was encountered in some samples (7.14%). The polyclonal infections constituting of MAD_{20}/RO33 were more

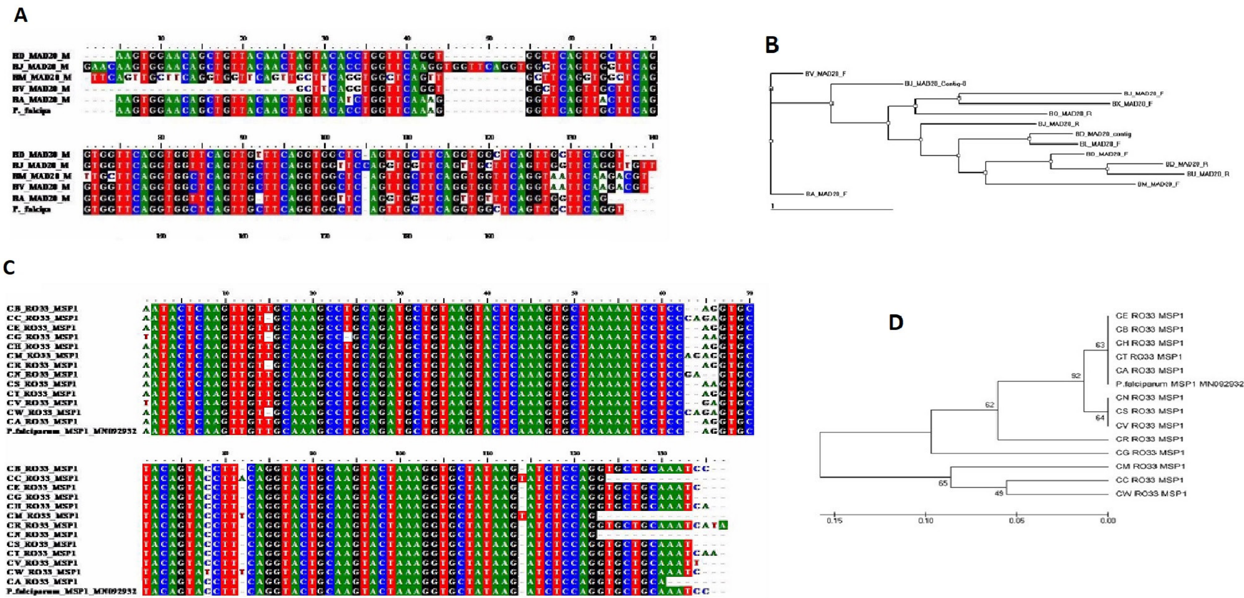


Figure 2: Multiple sequence alignment and Phylogenetic trees of *msp* 1 allelic families. MAD20 nucleotide sequences (A) and resultant phylogenetic tree (B) showing 4 categories of the 14 DNA sequences analysis with a 0.168 proportion of invariant. RO33 nucleotide sequences (C) and Phylogenetic tree (D) with four categories. Trees are drawn to scale with all gaps and missing data eliminated.

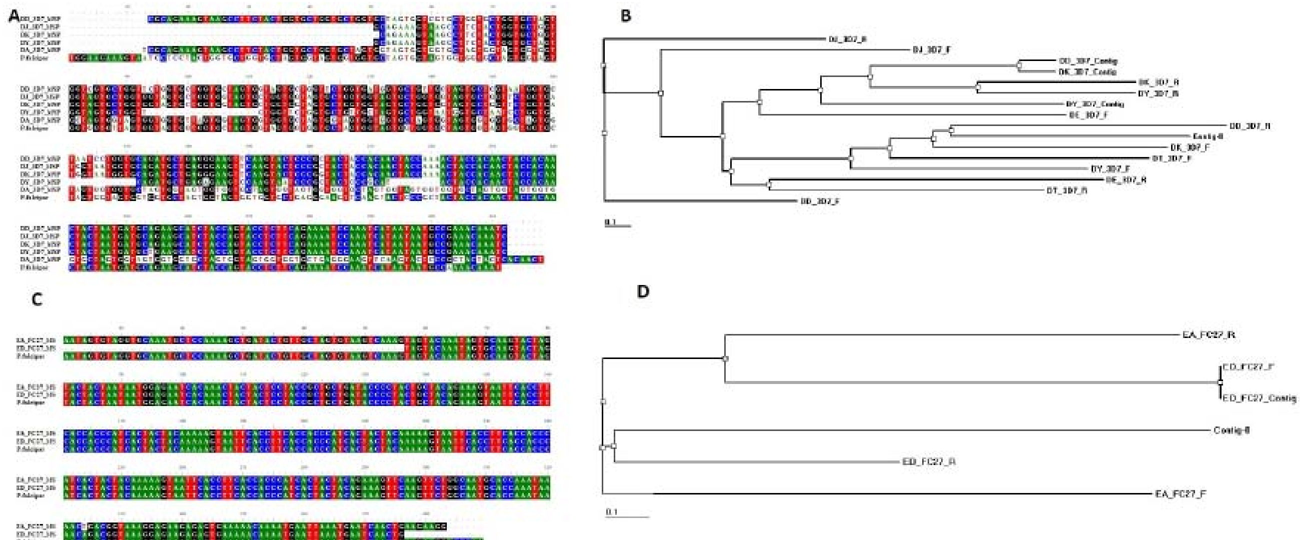


Figure 3: Multiple sequence alignment and Phylogenetic trees of msp 2 alleles. (A) 3D7 nucleotide and (B) Phylogenetic tree showing four categories with a 0.091 and 0.213 proportion of invariant. FC27 (C) allelic family, phylogenetic tree (D) also showing four categories with a 0.091 and 0.213 proportion of invariant. All positions with gaps or missing data were eliminated. Both trees are drawn to scale the same units used for branch length as well as inferring evolutionary distances.

(23.81%) than those of MAD_{20}/KI and KI/RO33 (Figure 1D). However, in the *msp 2* alleles, polyclonal infections constituting of 3D7/FC27 were few (22.6%) in number (Figure 1E). The multiplicity of infection (MOI) for *msp* 1 was seen to be higher (1.6) than that of *msp* 2 (1.32) as shown in Table 3.

Associated frequencies of alleles for *msp* 1 *and msp* 2 *P. falciparum* gene

There were nine observed alleles in *msp* 1 based on the results from PCR fragment amplification. For MAD_{20}, fragment sizes ranged between 200 – 225 bp, in KI fragment sizes ranged between 115 – 225 bp while only one fragment size of 150 bp was associate with RO33.

Table 4: Accession number, the strain, source of origin and reference of imported *Plasmodium falciparum malaria* cases.

Accession number	Strain	Origin	Reference
AB502545.1 AB502526.1 AB502597.1 AB502487.1 AB502528.1 AB502745.1 AB502678.1 AB502637.1 AB502443.1	msp 1: Gha1108-043 msp1: Gha1109-125 msp1: Pal97-039 msp1: Tz93-035 msp1: Gha1109-097 msp1: Sol95-121 msp1: PNG228-250 msp1: PNG904-130 msp1: Tz03-002	Ghana Ghana Central African Tanzania Ghana Solomon Islands Papua New Guinea Papua New Guinea Tanzania	[20]
AB827755.1 AB827752.1	msp1: PFS67 msp 1: PFS62	Thailand Thailand	[21]
AF509654.1 AF509696.1	msp 1Bl61 msp 1V49 partial	Viet Nam Viet Nam	[22]
HM568580.1 HM568568.1	isolate 280 partial cds isolate 216 partial cds	India	[23]
EU032224.1	*msp* 1: DM32D72, partial cds	Senegal	[24]
KR063228.1 KR063231.1	*msp* 1: PGIMER-2013-P16 PGIMER-2014-*msp* 1-P51.	India	[5]
HM153233.1	isolate 2/040-1-2 partial cds	Malawi	[25]
AB502626.1	Pal97-052	Philippines	[26]
JX412319.1	MAD_{20} isolate 48 partial cds	Western Brazilian Amazon	[27]
JF460903.1	RDTCF0177 *msp* 1, partial cds	India	[28]
DQ485450.1 AF217036.1	msp 1: Ke1, partial cds msp 2: 15-70A. partial cds	India Ghana	[29]
JX283499.1 JX885948.1 JX283523.1	msp 1: JGD162, partial cds msp 2: NF107, partial cds msp 2: JGD87, partial cds	Central India Thailand India	[30]
AJ318754.2	msp 2: partial 3D7-wos44	Papua New Guinea	[31]
LN999943.1 JX885946.1	3D7 chromosome 2 msp 2: A TC1125, partial cds	NI Thailand	[32]
DQ166542.1	msp 2: wos58 gene, partial cds	NI	[33]
AY378310.1	msp 2-O allele, partial cds	NI	[34]
JX885940.1	msp 2: CD037, partial cds	Thailand	[35]
KC887547.1	Sub clone MOA_day35 MSP 2	Gabon	[36]
(EMP1)			

NI = Not Indicated

Details of percentage distribution of clone sizes among the samples is shown in Figure 1F. The majority of *msp* 1 were the 150 bp RO33 allele and the 225 bp clone size of MAD20 allele. For *msp* 2, fifteen clones were encountered eleven of which were detected in the 3D7 allelic family with fragment sizes ranging between 175 – 375 bp (Figure 1G). Majority of the *msp* 2 alleles were 3D7 of which the dominate fragment size was 175 bp. There were 5 clones seen in FC27 allele with fragment sizes that ranged between 325 – 625 bp. In this group, clones with 405 bp fragment sizes were dominant (Figure 1G).

Diversity in *P. falciparum* nucleotide sequences of imported Malaria cases

The genetic similarities of alleles of *msp* 1 and 2 with those earlier reported in malaria endemic countries globally, were retrieved from NCBI databases. The GeneBank ascensions numbers https://www.ncbi.nlm.nih.gov/nucleotide of earlier reported sequences and region of isolation are presented in Table 4. For *msp* 1 allelic families, K1, MAD_{20}, RO33 haplotypes were found to be similar to those reported from other regions in countries that included Ghana, Central sub-Saharan African, Tanzania, Senegal, Malawi, Solomon

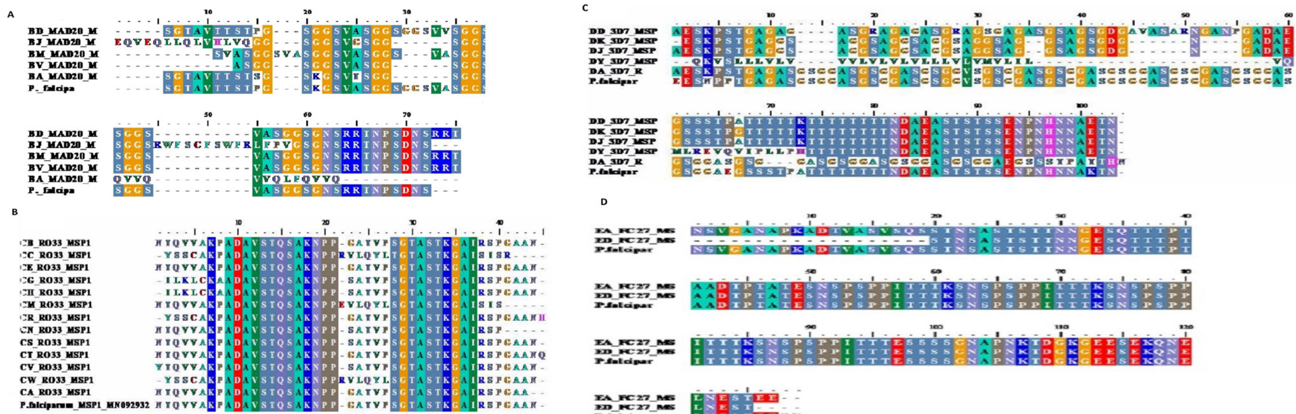
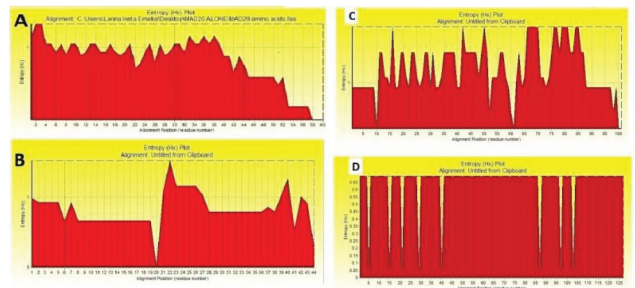


Figure 4: Amino acid sequence alignments of *Plasmodium falciparum*. MAD$_{20}$ (A) Distinct variants were found in the sequence analysis of msp 1-MAD$_{20}$ alleles as follows: 28 from BJ_MAD$_{20}$; 27 from BA_MAD$_{20}$; 19 from BM_MAD$_{20}$ and 3 from BV_MAD$_{20}$. In RO33 of msp 1 amino acid sequence (B), distinct variants were found by sequence analysis as follows: 15 from CC_RO33; 10 from CW_RO33; 7 from CG_RO33; 9 from CM_RO33; 5 from CR_RO33 msp 1; 2 from CV_RO33 and 1 each from CV_RO33; CS_ RO33 and CT_RO33. 7C - D: shows amino acid sequence alignment for msp 2 3D7 and FC27. Distinct variants were found by sequence analysis of msp 2-3D7 (C). The highest was 71 from DA_3D7; followed with 42 from DY_3D7; 11 and 10 from DK_3D7 and DJ_3D7 respectively. However, sequence alignments of amino acid of *msp* 2-FC27 distinct allelic variants of *Plasmodium falciparum* isolates of imported cases of malaria (D) exhibited minor variants. Variations in sequence analysis were as a result of deletions represented as dashes. Identical residues have been highlighted in colors.

Islands, Thailand Kanchanaburi Province, Vietnam, Philippines, Western Brazilian Amazon, Papua New Guinea and various regions of India (Table 4). For 3D7 and FC27 alleles of *msp* 2 *P. falciparum*, some of the haplotypes are similar to those retrieved from NCBI as follows: AJ318754.2 (Papua New Guinea); JX885948.1 (Thailand); AF217036.1 (Ghana); JX885948.1, JX885940.1; JX885946.1 (Thailand); JX283523.1 (India); KC887547.1 (Gabon). Others included LN999943.1 of 3D7 chromosome 2, DQ166542.1 isolate wos58 (*msp* 2) gene, and AY378310.1 *msp* 2-O allele, partial cds (Table 4).

Multiple sequence alignment (MSA) and phylogenetic analysis for *msp* 1 *alleles of P. falciparum* gene

Three alleles, KI, MAD$_{20}$ and RO33 were sequenced and analysed for *msp 1 P. falciparum* gene (SS 3 A – C). In KI allelic family, four DNA nucleotide sequences of 114 positions, which constituted 15% of the original 736 positions, were analysed. No gap positions were allowed with the obtained flanked positions of 8 selected blocks, namely75-84, 137-146, 264-279, 381-394, 533-561, 576-587, 609-618 and 697-709. In addition, the obtained nucleotides frequencies for the KI alleles were as follows: Adenine (A) 0.36842, Cytosine (C) 0.16667, Guanine (G) 0.17763 and Thymine (T) 0.28728. For MAD$_{20}$ allele of *msp* 1 *P. falciparum* gene, six nucleotide sequences were analysed of which positions with gaps and missing data were eliminated (Figure 2A). A final data set of 96 positions were obtained with flank positions of three selected blocks 45-103, 123-140 and 153-191.



Supplementary Figure 1: Entropy (HX) plot for *Plasmodium falciparum* nucleotides (A-D). MAD20 (A) and RO33 (B) msp 1 amino acid nucleotide of *Plasmodium falciparum* of imported malaria case. 688 Also, Entropy [Hx] plot of amino acids sequence of 3D7 (C) and FC27 (D) msp 2 *Plasmodium falciparum* gene. Plots were obtained using BioEdit version 7 to analyse amino acid nucleotide sequences with entropy scores for individual positions shown in the Y-axis while the X-axis 691 show residue positions for multiple sequence alignment [MSA]. Entropy [Hx] flanked positions varied between high conservation (0) to hypervariable (1.0).

Obtained nucleotide frequencies were as follows: Adenine (A) 0.20690, Cytosine (C) 0.17672, Guanine (G) 0.28233 and Thymine (T) 0.33405. An optimal tree with 0.00313072 branch length is shown in Figure 2B with associated samples clustered together in bootstrap test indicated while evolutionary analysis was carried out using MEGA version 4.

Multiple sequence alignment analysis for RO33 allelic family for *msp* 1 *Plasmodium falciparum* gene (Figure 2C) was of fourteen nucleotide sequences analysed using MEGA

version 4 with a total of 114 positions in the final data set.[37] The obtained nucleotide frequencies for this allelic family were A (0.31923), C (0.17179), G (0.24744) and T (0.26154). The optimal tree with the sum branch length of 0.01764909 was inferred using the neighbour-joining method previously described.[38] Associated taxa clustered together of replicate trees are indicated according to the confidence limits on phylogenies by Felsenstein.[39] The tree drawn to scale is shown in Figure 2D.

Multiple sequence alignment (MSA) and phylogenetic analysis for *msp* 2 alleles of *P. falciparum* gene

MSA for *msp 2 P. falciparum* gene involved the 3D7 and FC27 allelic families. For 3D7 allele of *msp 2* analysis involved six nucleotide sequences (Figure 3A) with all gaps or missing data eliminated. Flanked positions of eight selected blocks 1-24, 30-98, 107-119, 127-148, 151-187, 225-241, 272-285, 288-304 were obtained. There were a total of 222 positions of which nucleotide frequencies were as follows: (A) 0.25822, (C) 0.21596, (G) 0.24178 and (T) 0.28404. The neighbouring joining method[38] was used for inferring the phylogenetic trees of 3D7 and FC27 allelic families of the *msp 2* gene (Figures 3B, 3C). Three nucleotide sequences were analysed with 320 positions in the final data set for the FC27 allele of *msp* 2 *P. falciparum* gene (SS 4B). Flanked positions of four selected blocks 79-95, 102-120, 126-148, and 160-207 were obtained with nucleotide frequencies of A (0.31923), C (0.17179), G (0.24744), and T (0.26154).

Entropy plots for allelic families of *msp* 1 and *msp* 2 *P. falciparum* gene

Entropy plots used to measure the amount of variability in the amino acids for merozoite proteins 1 and 2 are shown in Supplementary Figure 1. Entropy Hx plots were obtained with BioEdit version 7 with values defined as described earlier.[40] For *msp* 1, entropy plots showed minimal entropy with most positions barely getting to the scale of one (Figure S1 A, B). Similar observation was seen with *msp* 2 3D7 and FC27 amino acids entropy plot. However, the entropy [Hx] for FC27-*msp* 2, is seen to be below the scale of one being signs of high similarities with lower entropy implying the most conserved regions (Figure S1 C, D).

Genetic variations in amino acid of alleles of *msp* 1 and *msp* 2 *P. falciparum*

Tripeptide repeat units varied in the sequences of *msp* 1 and *msp* 2 as shown in Figure 4 A-D. In the MAD_{20} allelic type, the tripeptide alleles starts with SGG, and ends with two tripeptide units (SGGSAV/DNSRRT). The different tripeptide repeat units are, SGG, SGT, SVA, SGN, SRR, and TNP. The allelic diversity as seen in this group (Figure 4A), can be attributed to substitutions and deletions in these motifs. Allelic diversity in RO33-*msp* 1 amino acid sequence showed that for this allelic type, the tripeptide region started with NTQ and ended with AAN. Diversity in this allele can therefore be attributed to both deletions and substitution in repeat motif in the (NTQVVAKPADAVSTQSAK-NPP/GATVPSGTASTKGAIRSPGAAN) sequence as shown (Figure 4B).

For *msp* 2 3D7 allelic variants of imported *P. falciparum* malaria cases, tripeptide re-peat units starts with four tripeptide units, AES, KPS, TGA and GAS (Figure 4C). Genetic variations are attributed to duplications, deletions as well as substitutions in the repeat motifs. Sequence alignments of amino acid sequences of *msp* 2-FC27 distinct allelic variants among *Plasmodium falciparum* isolates showed no distinct variants by sequence analysis (Figure 4D).

DISCUSSION

The genetic diversity of imported malaria parasites impacts malaria control strategies and hence portends danger of recrudescence.[41] From the foregoing, the identification of population structure of migrants bringing in malarial parasites associated endermicity, should be a public health objective. In the present study, we examined the genetic diversity of imported *P. falciparum*. We also studied their clonal assortments in terms of associated population structure of these imported cases. Our study comes on the wake of the fact that Saudi Arabia has a large dynamic migrant workforce therefore, imported malaria could underscore their control measures. Demographic results showed infections were from children and adults with history of travel into and within the Kingdom, calls for the need for a continuation in stringent control measures if there is to be a complete eradication of imported malaria cases. However the small sample size collected over a period of two years could be possible pointers to attained success so far in this region of study that will need to be maintained if Saudi Arabia is to retain its status as a "country on the verge of malaria elimination".[42,43] On the other hand, based on WHO (2008) recommendation[44] of *msp* 1 and *msp* 2 being standard markers in distinguishing recrudescence from newly infecting parasite of malaria, the present investigation evaluated genetic diversity in these allelic families of *P. falciparum* gene. The obtained results which showed genotypes of *msp* 1

predominating those of *msp* 2 with differing genetic polymorphism, are similar to those reported from disease endemic countries.[17,45]

The study also showed that RO33 were encountered in all the samples thus predominating MAD_{20} and KI in the allelic families of the *msp* 1 *P. falciparum* gene. These findings are similar to those of earlier reports.[46-48] However, contrary to this are other reports in which KI and MAD_{20} *msp* 1 were found to predominate RO33.[17] Thus, there are differences in the predominance of alleles which could be attributed to a number of suggested reasons. One of such is that KI allele dominance was associated with severe malaria,[17] while others associated this dominance with asymptomatic malaria.[49,50] The present study did not look into the severity or otherwise of symptoms of malaria in the patients from where the samples had been taken. Neither could the infections be said to be asymptomatic. Predominance of RO33 could possible simply be a reflection of that obtainable from the origin of travel into Saudi Arabia. In the *msp* 2 alleles, FC27 predominated 3D7 in this study with the findings similar to those of earlier reports in disease endemic regions.[51-54] However, while 3D7 allele was predominant in countries like Pakistan, Papua New Guinea, Thailand,[55-57] FC27 was predominant in reports from countries such as Nigeria, Cameroon and Gabon.[57-59] Thus predominance of FC27 in this study could again be a reflection of the origin of travel into the Kingdom. Considering the fact that majority of the imported cases here were from African countries of Sudan and Nigeria, there is therefore the possibility that predominance in alleles of either *msp* 1 or *msp* 2, could be attributed to geographical regions of travel origin. On the other hand, FC27-*msp* 2 allele had been generally associated with severity of malaria while it is thought that the 3D7-*msp* 2 is common in areas where transmission of malaria is high. Thus, while there is no such transmission in this region of Southeast Saudi Arabia, results in this report again could be reflecting region of travel of the imported cases. Some authors[60] did however suggest that dominance of some alleles could either be accidental or could be due to a stable life cycle of *P. falciparum* parasite. There is the possibility that low *P. falciparum* number of clones seen here are as a result of a restricted pool of genes as earlier suggested.[60,61] However, there were diversities generally in the genotypes of *msp* 1 and *msp* 2 *P. falciparum* gene in this investigation. By PCR agarose visualisation (supplementary data Figure 1, 2 and 3) diversity seen among the clones of alleles of *msp* 1 (9) and *msp* 2 (11) cannot be described as high when compared with those seen in other reports.[45,49] High diversity those reports had attributed to the intensity of malaria transmission as well as exposures to multiple parasite infections carrying different genotypes. This could explain the reason for the results here of the imported malaria samples with similar findings having been reported previously.[62-64] There is also the possibility of treatment with antimalarial before travelling into the Kingdom and this could reflect in the results. Also, MOI of the imported cases of malaria in this region of study was low when compared with those of other findings.[65] This is a positive direction with a possibility of reduced risk for parasite transmission. On the other hand, similar to our findings are those of Somé *et al.*,[17] in which low MOI had been attributed to possible success of malaria control measures. Others had associated high values of MOI with hyper endemic regions where there was high transmission of the disease.[55,66] However, the samples here are imported into a southeast region of the Kingdom with no active parasite transmission. Also worthy of note are the polyclonal samples seen in this study. Such infections are reportedly common in high endemic regions.[64,67] The results here, are therefore not unexpected when considering the origins of the samples. Similarly, nucleotide sequences of *msp* 1 and *msp* 2 allelic families found here are similar to those found in other regions of the world. Phylogenetic tree analysis of sequenced KI *msp* 1 grouped this allele into four categories. Three haplotypes of which two (AB502545, AB502526), were 95 and 97 % in similarity as those reported in Ghana[20] while the other (AB827755) was 98 % similar to that reported in Thailand.[21] Also, the KI-*msp* 1 tripeptide motifs (SAQ, SGA, SGT, SGP) which were seen in combination with tripeptides (SGASAQ, SGTSGP, SAQSGA) reported in this study are similar to other documented findings.[68] Thus, this pattern of tripeptide motifs and their combinations here could be associated with African allele trend as earlier suggested.[68] For MAD_{20}-*msp* 1, genetic diversity was detected in the number of variants obtained from analysed sequences of this allele. Four variants (HM568580, KR063228, and HM568568) seen here, are between 94 – 99% similar to those in India.[25,27] One variant (AB502745) with 100 % similarity had been reported in Solomon Island,[20] while others variants with 99 % similarity, in Tanzania (AB502487) Central Africa (AB502597), Senegal (EUO32224), and Ghana (AB502528, AB502626).[20,24] There were also other variants from Vietnam (AF509654, AF509696),[22] Thailand (AB8227752),[21] Western Brazilian Amazon (JX412319)[27] and Papua New Guinea (AB502678, AB502637).[20] Worthy of note is that some of the listed countries are not among the listed for imported cases of malaria to the region of the present study and could

only be explained to be due to human movement to different regions of the world. Also, the phylogenetic of analysed sequence of RO33 showed four categories variants of which had been reported by other researchers in India (KR063231),[5]

JF460903,[28] DQ485450,[29] JX283499[30] and Tanzania (AB502443).[20] For 3D7-*msp* 2, there were nine variants in imported malaria case in this study which are similar to those from Thailand (JX885948, JX885946, JX885940),[30,32,35] Papua New Guinea (AJ318754)[31] and Ghana (AF217036)[29] had been reported. There were other variants (LN99994,

DQ166542, and AY378310) that were reportedly deposited with NCBI by Welcome Trust UK. On the other hand however, only one haplotype (KC887547) with 99 % similarity to that reported in Gabon was detected in FC27-*msp* 2.[36]

CONCLUSION

This study described the genetic diversity of *P. falciparum* and their population structure of imported cases of malaria into South East region of Saudi Arabia during 2018–2020. Genotyping of *msp* 1 and *msp* 2 of *P. falciparum* revealed varying levels of genetic and clonal diversity. Furthermore, our findings show that the population structure of imported *P. falciparum* parasites appeared to exhibit high diversity as seen from the allelic families of sequenced data. The findings highlight the possible threat posed by cases of imported malaria to the success achieved by Saudi Arabia in the elimination of the disease as had earlier been highlighted. The yet to be attained malaria free status planned for 2020 will need continuous monitoring.

Funding

The research was funded by a grant from Deanship of Scientific Research, King Faisal University, Al-Ahsa, Saudi Arabia. Grant number 160107.

ACKNOWLEDGEMENT

Authors acknowledge Deanship for Scientific research, King Faisal University, Al-Ahsa for financing of the research. We would like to thank Dr. Promise Emeka and Dr. Krishnaraj Thirugnanasambantham for their help with data analysis. We thank Hani Al-Farhan, Hani Al-Rasasi and Fatimah Al-Najja for their technical support. We would also like to acknowledge BEI Resources, 10801 University Boulevard Manassas, VA 20110-2209 for providing us with MRA-102G Genomic DNA 3D7 *Plasmodium falciparum* and MRA-155G Genomic DNA from HB3 *Plasmodium falciparum*.

ABBREVIATIONS

MOI: Multiplicity of infection; **nPCR:** nested PCR; **PCR:** Polymerase chain reaction; **DBS:** Dried blood spots; **msp:** merozoite protein.

CONFLICT OF INTEREST

The authors declare there are no conflict of interest.

REFERENCES

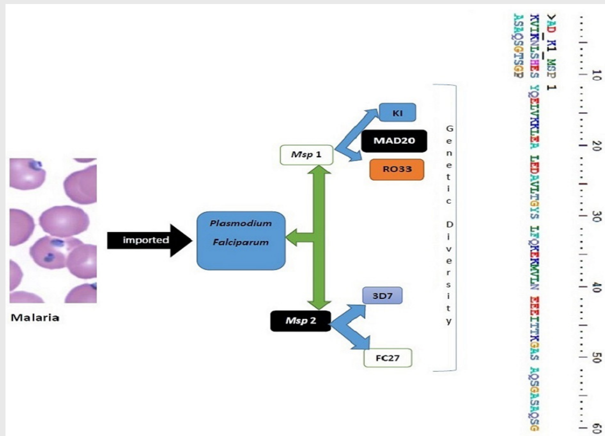
1. Piperaki ET, Daikos GL. Malaria in Europe: emerging threat or minor nuisance? Clin Microbiol Infect. 2016 Jun;22(6):487-93. doi: 10.1016/j.cmi.2016.04.023, PMID 27172807.
2. Smith DL, Cohen JM, Chiyaka C, Johnston G, Gething PW, Gosling R, Buckee CO, Laxminarayan R, Hay SI, Tatem AJ. A sticky situation: the unexpected stability of malaria elimination. Philos Trans R Soc Lond B Biol Sci. 2013 Aug 5;368(1623):20120145. doi: 10.1098/rstb.2012.0145.
3. The "World malaria report at a glance. World Health Organization; 2019. Available from: https://www.who.int/news-room/feature-stories/detail/world-malaria-report-2019.
4. Mischlinger J, Rönnberg C, Álvarez-Martínez MJ, Bühler S, Paul M, Schlagenhauf P, et al. Imported malaria in countries where malaria is not endemic: a comparison of semi-immune and nonimmune travelers. Clin Microbiol Rev. 2020 03 18;33(2):e00104-19. doi: 10.1128/CMR.00104-19, PMID 32161068.
5. Al Zahrani MH, Omar AI, Abdoon AMO, Ibrahim AA, Alhogail A, Elmubarak M, Elamin YE, AlHelal MA, Alshahrani AM, Abdelgader TM, Saeed I, El Gamri TB, Alattas MS, Dahlan AA, Assiri AM, Maina J, Li XH, Snow RW. Cross-border movement, economic development and malaria elimination in the Kingdom of Saudi Arabia. BMC Med. 2018;16(1):98 26. doi: 10.1186/s12916-018-1081-z, PMID 29940950.
6. Coleman M, Al-Zahrani MH, Coleman M, Hemingway J, Omar A, Stanton MC, Thomsen EK, Alsheikh AA, Alhakeem RF, McCall PJ, Al Rabeeah AA, Memish ZA. A country on the verge of malaria elimination – the Kingdom of Saudi Arabia. PLOS ONE. 2014;9(9):e105980. doi: 10.1371/journal.pone.0105980, PMID 25250619.
7. World Health Organization. World malaria report. Geneva; 2011. Available from: https://www.who.int/malaria/world_malaria_report_2011/9789241564403_eng.pdf [cited 16/9/2021].
8. Khan AS, Qureshi F, Shah AH, Malik SA. Spectrum of malaria in Hajj pilgrims in the year 2000. J Ayub Med Coll Abbottabad. 2002 Oct–Dec;14(4):19-21. PMID 12688096.
9. Soliman RH, Garcia-Aranda P, Elzagawy SM, Hussein BE, Mayah WW, Ramirez M. de Roode JC, Pansini R, Cheesman SJ, Helinski ME, Huijben S, Wargo AR, Bell AS, Chan BH, Walliker D, Read AF. Virulence and competitive ability in genetically diverse malaria infections. Proc Natl Acad Sci U S A. 2005 May 24;102(21):7624-8. doi: 10.1073/pnas.0500078102, PMID 15894623.
10. Read AF, Taylor LH. The ecology of genetically diverse infections. Science. 2001 May 11;292(5519):1099-102. doi: 10.1126/science.1059410, PMID 11352063.
11. Lalremruata A, Jeyaraj S, Engleitner T, Joanny F, Lang A, Bélard S, Mombo-Ngoma G, Ramharter M, Kremsner PG, Mordmüller B, Held J. Species and genotype diversity of Plasmodium in malaria patients from Gabon analysed by next generation sequencing. Malar J. 2017;16(1 (1)):398. doi: 10.1186/s12936-017-2044-0, PMID 28974215.
12. Juliano JJ, Porter K, Mwapasa V, Sem R, Rogers WO, Ariey F, Wongsrichanalai C, Read A, Meshnick SR. Exposing malaria in-host diversity and estimating population diversity by capture-recapture using massively parallel pyrosequencing. Proc Natl Acad Sci U S A. 2010;107(46):20138-43. doi: 10.1073/pnas.1007068107, PMID 21041629.
13. Preparation of Blood Spots on filter paper. WHO.; 2016. Available from: https://apps.who.int/iris/bitstream/handle/10665/274382/MM-SOP-10-eng.pdf?sequence=15&isAllowed=y [cited 16/9/2021].

14. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Mol Biochem Parasitol. 1993 Apr;58(2):283-92. doi: 10.1016/0166-6851(93)90050-8, PMID 8479452.
15. University of Maryland School of Medicine. Center for Vaccine Development. Malaria Group. University of Maryland; 2017, Aug 22. Protocol for MSP-2 Genotyping. Available from: https://archive.hshsl.umaryland.edu/handle/10713/7040 [cited 16/9/2021].
16. Somé AF, Bazié T, Zongo I, Yerbanga RS, Nikiéma F, Neya C, Taho LK, Ouédraogo JB. *Plasmodium falciparum* msp1 and msp2 genetic diversity and allele frequencies in parasites isolated from symptomatic malaria patients in Bobo-Dioulasso, Burkina Faso. Parasit Vectors. 2018 May 30;11(1):323. doi: 10.1186/s13071-018-2895-4, PMID 29843783.
17. Abukari Z, Okonu R, Nyarko SB, Lo AC, Dieng CC, Salifu SP, et al. The diversity, multiplicity of infection and population structure of *P. falciparum* parasites circulating in asymptomatic carriers living in high and low malaria transmission settings of Ghana. Genes (Basel). 2019;10(6):E434:06 7. doi: 10.3390/genes10060434, PMID 31181699.
18. Mahdi Abdel Hamid M, Elamin AF, Albsheer MM, Abdalla AA, Mahgoub NS, Mustafa SO, Muneer MS, Amin M. Multiplicity of infection and genetic diversity of *Plasmodium falciparum* isolates from patients with uncomplicated and severe malaria in Gezira State, Sudan. Parasit Vectors. 2016;9(1):362. doi: 10.1186/s13071-016-1641-z. PMID 27350250.
19. Tanabe K, Mita T, Palacpac NM, Arisue N, Tougan T, Kawai S, Jombart T, Kobayashi F, Horii T. Within-population genetic diversity of *Plasmodium falciparum* vaccine candidate antigens reveals geographic distance from a Central sub-Saharan African origin. Vaccine. 2013 Feb 18;31(9):1334-9. doi: 10.1016/j.vaccine.2012.12.039, PMID 23295064.
20. Tanabe K, Zollner GE, Sattabongkt J, Khuntirat B, Honma H, Mita T, Tsuboi T, Coleman R. Genetic diversity of *Plasmodium falciparum* in an isolated village in western Thailand; unpublished.
21. Ferreira MU, Ribeiro WL, Tonon AP, Kawamoto F, Rich SM. Sequence diversity and evolution of the malaria vaccine candidate merozoite surface protein-1 (MSP-1) of *Plasmodium falciparum*. Gene. 2003 Jan 30;304:65-75. doi: 10.1016/s0378-1119(02)01180-0, PMID 12568716.
22. Sharma YD. Genetic diversity of *Plasmodium falciparum* vaccine candidate antigens from Madhya Pradesh, India; unpublished.
23. Noranate N, Prugnolle F, Jouin H, Tall A, Marrama L, Sokhna C, Ekala MT, Guillotte M, Bischoff E, Bouchier C, Patarapotikul J, Ohashi J, Trape JF, Rogier C, Mercereau-Puijalon O. Population diversity and antibody selective pressure to *Plasmodium falciparum* msp 1 block2 locus in an African malaria-endemic setting. BMC Microbiol. 2009;9:219. doi: 10.1186/1471-2180-9-219, PMID 19832989.
24. Sehgal R, Kaur H, Bansal D, Sultan AA. Genetic diversity of *Plasmodium falciparum* Merozoite Surface Protein 1 (block2 region) from Chandigarh and adjoining states of North India; unpublished.
25. Jombart T, Kobayashi F, Horii T. Within-population genetic diversity of *Plasmodium falciparum* vaccine candidate antigens reveals geographic distance from a Central sub-Saharan African origin Vaccine. 2013;31(9):1334-9.
26. Medeiros MM, Fotoran WL, dalla Martha RC, Katsuragawa TH, Pereira da Silva LH, Wunderlich G. Natural antibody response to *Plasmodium falciparum* merozoite antigens MSP5, MSP9 and EBA175 is associated to clinical protection in the Brazilian Amazon. BMC Infect Dis. 2013 Dec 28;13:608. doi: 10.1186/1471-2334-13-608, PMID 24373342.
27. Bharti PK, Shukla MM, Sharma YD, Singh N. Genetic diversity in the block 2 region of the merozoite surface protein-1 of *Plasmodium falciparum* in central India. Malar J. 2012 Mar 22;11:78. doi: 10.1186/1475-2875-11-78, PMID 22439658.
28. Joshi H, Valecha N, Verma A, Kaul A, Mallick PK, Shalini S, Prajapati SK, Sharma SK, Dev V, Biswas S, Nanda N, Malhotra MS, Subbarao SK, Dash AP. Genetic structure of *Plasmodium falciparum* field isolates in eastern and north-eastern India. Malar J. 2007;6:60. doi: 10.1186/1475-2875-6-60, PMID 17517129.
29. Singh PP, Bharti PK, Sawale PT, Singh N. Antigenic variations and multiplicity of infection of *Plasmodium falciparum* malaria in peripheral and placental samples from Central India; unpublished.
30. Flück C, Schöpflin S, Smith T, Genton B, Alpers MP, Beck HP, Felger I. Effect of the malaria vaccine Combination B on merozoite surface antigen 2 diversity. Infect Genet Evol. 2007 Jan;7(1):44-51. doi: 10.1016/j.meegid.2006.03.006, PMID 16647307.
31. Chaorattanakawee S, Nuchnoi P, Hananantachai H, Tumkosit U, Naka I, Ohashi J, Patarapotikul J. Variation of merozoite surface protein-2 of *Plasmodium falciparum* is associated with virulence causing cerebral malaria; unpublished.
32. Franks S, Koram KA, Wagner GE, Tetteh K, McGuinness D, Wheeler JG, Nkrumah F, Ranford-Cartwright L, Riley EM. Frequent and persistent, asymptomatic *Plasmodium falciparum* infections in African infants, characterized by multilocus genotyping. J Infect Dis. 2001 Mar 1;183(5):796-804. doi: 10.1086/318834, PMID 11181157.
33. Gardner MJ, Tettelin H, Carucci DJ, Cummings LM, Aravind L, Koonin EV, Shallom S, Mason T, Yu K, Fujii C, Pederson J, Shen K, Jing J, Aston C, Lai Z, Schwartz DC, Pertea M, Salzberg S, Zhou L, Sutton GG, Clayton R, White O, Smith HO, Fraser CM, Adams MD, Venter JC, Hoffman SL. Chromosome 2 sequence of the human malaria parasite Plasmodium falciparum. Science. 1998 Nov 6;282(5391):1126-32. doi: 10.1126/science.282.5391.1126, PMID 9804551.
34. Felger I, Steiger S, Hatz C, Smith T, Beck HP. Antigenic cross-reactivity between different alleles of the *Plasmodium falciparum* merozoite surface protein 2. Parasite Immunol. 2003 Nov–Dec;25(11-12):531-43. doi: 10.1111/j.0141-9838.2004.00664.x, PMID 15053774.
35. Enderes C, Bruske EI, Tschan S, Kombila D, Kremsner P, Frank M. *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is not the main variant surface antigen of field isolates from a chronic asymptomatic infection; unpublished.
36. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol software version 4.0. 2007 Aug;24(8):1596-9. doi: 10.1093/molbev/msm092, PMID 17488738.
37. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987 Jul;4(4):406-25. doi: 10.1093/oxfordjournals.molbev.a040454, PMID 3447015.
38. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985 Jul;39(4):783-91. doi: 10.1111/j.1558-5646.1985.tb00420.x, PMID 28561359.
39. Tripathi V, Gupta D. Evolutionary analysis of circumsporozoite surface protein and merozoite surface protein-1 (CSP and MSP-1) sequences of malaria parasites. Bioinformation. 2011;6(8):320-3. doi: 10.6026/97320630006320, PMID 21769195.
40. Soe TN, Wu Y, Tun MW, Xu X, Hu Y, Ruan Y, Win AYN, Nyunt MH, Mon NCN, Han KT, Aye KM, Morris J, Su P, Yang Z, Kyaw MP, Cui L. Genetic diversity of *Plasmodium falciparum* populations in southeast and western Myanmar. Parasit Vectors. 2017 Jul 4;10(1):322. doi: 10.1186/s13071-017-2254-x, PMID 28676097.
41. Dhiman S. Are malaria elimination efforts on right track? An analysis of gains achieved and challenges ahead. Infect Dis Poverty. 2019 Feb 13;8(1):14. doi: 10.1186/s40249-019-0524-x, PMID 30760324.
42. El Hassan IM, Sahly A, Alzahrani MH, Alhakeem RF, Alhelal M, Alhogail A, Alsheikh AA, Assiri AM, ElGamri TB, Faragalla IA, Al-Atas M, Akeel MA, Bani I, Ageely HM, BinSaeed AA, Kyalo D, Noor AM, Snow RW. Progress toward malaria elimination in Jazan Province, Kingdom of Saudi Arabia: 2000-2014. Malar J. 2015 Nov 9;14:444. doi: 10.1186/s12936-015-0858-1, PMID 26552387.
43. World Health Organization. Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite population. Geneva: World Health Organization; 2008. Available from: https://www.who.int/malaria/publications/atoz/9789241596305/en/ [cited 16/9/2021].
44. Soulama I, Nébié I, Ouédraogo A, Gansane A, Diarra A, Tiono AB, Bougouma EC, Konaté AT, Kabré GB, Taylor WR, Sirima SB. *Plasmodium falciparum* genotypes diversity in symptomatic malaria of children living in an urban and a rural setting in Burkina Faso. Malar J. 2009 Jun 20;8:135. doi: 10.1186/1475-2875-8-135, PMID 19545390.
45. Huang B, Tuo F, Liang Y, Wu W, Wu G, Huang S, Zhong Q, Su XZ, Zhang H, Li M, Bacar A, Abdallah KS, Mliva AMSA, Wang Q, Yang Z, Zheng S, Xu Q,

Song J, Deng C. Temporal changes in genetic diversity of msp-1, msp-2, and msp-3 in *Plasmodium falciparum* isolates from Grande Comore Island after introduction of ACT. Malar J. 2018;17(1):83. doi: 10.1186/s12936-018-2227-3, PMID 29458365.

46. Ahmedou Salem MSO, Ndiaye M, OuldAbdallahi M, Lekweiry KM, Bogreau H, Konaté L, Faye B, Gaye O, Faye O, Mohamed Salem O Boukhary AO. Polymorphism of the merozoite surface protein-1 block 2 region in *Plasmodium falciparum* isolates from Mauritania. Malar J. 2014;13:26. doi: 10.1186/1475-2875-13-26, PMID 24456636.
47. Kiwuwa MS, Ribacke U, Moll K, Byarugaba J, Lundblom K, Färnert A, Fred K, Wahlgren M. Genetic diversity of *Plasmodium falciparum* infections in mild and severe malaria of children from Kampala, Uganda. Parasitol Res. 2013 Apr;112(4):1691-700. doi: 10.1007/s00436-013-3325-3, PMID 23408340.
48. Amodu OK, Adeyemo AA, Ayoola OO, Gbadegesin RA, Orimadegun AE, Akinsola AK, Olumese PE, Omotade OO. Genetic diversity of the msp-1 locus and symptomatic malaria in south-west Nigeria. Acta Trop. 2005 Sep;95(3):226-32. doi: 10.1016/j.actatropica.2005.06.017, PMID 16023985.
49. Babiker HA. Unstable malaria in Sudan: the influence of the dry season. *Plasmodium falciparum* population in the unstable malaria area of eastern Sudan is stable and genetically complex. Trans R Soc Trop Med Hyg. 1998 Nov–Dec;92(6):585-9. doi: 10.1016/s0035-9203(98)90774-x, PMID 10326096.
50. Mohammed H, Hassen K, Assefa A, Mekete K, Tadesse G, Taye G, Commons RJ. Genetic diversity of *Plasmodium falciparum* isolates from patients with uncomplicated and severe malaria based on msp-1 and msp-2 genes in Gublak, North West Ethiopia. Malar J. 2019 Dec 10;18(1):413. doi: 10.1186/s12936-019-3039-9, PMID 31823778.
51. Yavo W, Konaté A, Mawili-Mboumba DP, Kassi FK, Tshibola Mbuyi ML, Angora EK, Menan EI, Bouyou-Akotet MK. Genetic Polymorphism of msp1 and msp2 in *Plasmodium falciparum* Isolates from Côte d'Ivoire versus Gabon. J Parasitol Res. 2016;2016:3074803. doi: 10.1155/2016/3074803.
52. Mwingira F, Nkwengulila G, Schoepflin S, Sumari D, Beck HP, Snounou G, Felger I, Olliaro P, Mugittu K. *Plasmodium falciparum* msp1, msp2 and glurp allele frequency and diversity in sub-Saharan Africa. Malar J. 2011 Apr 6;10:79. doi: 10.1186/1475-2875-10-79, PMID 21470428.
53. Hamid MM, Mohammed SB, El Hassan IM. Genetic diversity of *Plasmodium falciparum* Field isolates in central Sudan inferred by PCR genotyping of merozoite surface Protein 1 and 2. N Am J Med Sci. 2013 Feb;5(2):95-101. doi: 10.4103/1947-2714.107524, PMID 23641369.
54. Ghanchi NK, Mårtensson A, Ursing J, Jafri S, Bereczky S, Hussain R, Beg MA. Genetic diversity among *Plasmodium falciparum* field isolates in Pakistan measured with PCR genotyping of the merozoite surface protein 1 and 2. Malar J. 2010 Jan 1;9:1. doi: 10.1186/1475-2875-9-1, PMID 20043863.
55. Snounou G, Zhu X, Siripoon N, Jarra W, Thaithong S, Brown KN, Viriyakosol S. Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. Trans R Soc Trop Med Hyg. 1999 Jul–Aug;93(4):369-74. doi: 10.1016/s0035-9203(99)90120-7, PMID 10674079.
56. Oyedeji SI, Awobode HO, Anumudu C, Kun J. Genetic diversity of *Plasmodium falciparum* isolates from naturally infected children in north-central Nigeria using the merozoite surface protein-2 as molecular marker. Asian Pac J Trop Med. 2013 Aug;6(8):589-94. doi: 10.1016/S1995-7645(13)60102-9, PMID 23790328.
57. Njama-Meya D, Kamya MR, Dorsey G. Asymptomatic parasitaemia as a risk factor for symptomatic malaria in a cohort of Ugandan children. Trop Med Int Health. 2004 Aug;9(8):862-8. doi: 10.1111/j.1365-3156.2004.01277.x, PMID 15303990.
58. Issifou S, Rogier C, Adjagba-Olakpo M, Chabi-Worou N, Ntoumi F. Complexity and genetic diversity of *Plasmodium falciparum* infections in young children living in urban areas of Central and West Africa. Parasitol Res. 2003 Aug;90(5):423-8. doi: 10.1007/s00436-003-0854-1, PMID 12759745.
59. Eida OM, Eida AM. Limited genetic diversity among Plasmodium falciparium isolates using nested PCR in Jazan Area, Saudi Arabia. Asian Pac J Trop Biomed. 2015;5(5):407-11. doi: 10.1016/S2221-1691(15)30377-4.
60. Babiker HA, Walliker D. Current views on the population structure of *Plasmodium falciparum*: implications for control. Parasitol Today. 1997 Jul;13(7):262-7. doi: 10.1016/s0169-4758(97)01075-2, PMID 15275063.
61. Duah NO, Matrevi SA, Quashie NB, Abuaku B, Koram KA. Genetic diversity of *Plasmodium falciparum* isolates from uncomplicated malaria cases in Ghana over a decade. Parasit Vectors. 2016;9(1):416. doi: 10.1186/s13071-016-1692-1, PMID 27460474.
62. Miotto O, Almagro-Garcia J, Manske M, Macinnis B, Campino S, Rockett KA, et al. 636. Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. Nat Genet. 2013 Jun;45(6):648-55. doi: 10.1038/ng.2624, PMID 23624527.
63. Yuan L, Zhao H, Wu L, Li X, Parker D, Xu S, Zhao Y, Feng G, Wang Y, Yan G, Fan Q, Yang Z, Cui L. *Plasmodium falciparum* populations from northeastern Myanmar display high levels of genetic diversity at multiple antigenic loci. Acta Trop. 2013 Jan;125(1):53-9. doi: 10.1016/j.actatropica.2012.09.008, PMID 23000544.
64. Vafa M, Troye-Blomberg M, Anchang J, Garcia A, Migot-Nabias F. Multiplicity of *Plasmodium falciparum* infection in asymptomatic children in Senegal: relation to transmission, age and erythrocyte variants. Malar J. 2008 Jan 23;7:17. doi: 10.1186/1475-2875-7-17, PMID 18215251.
65. Agyeman-Budu A, Brown C, Adjei G, Adams M, Dosoo D, Dery D, Wilson M, Asante KP, Greenwood B, Owusu-Agyei S. Trends in multiplicity of *Plasmodium falciparum* infections among asymptomatic residents in the middle belt of Ghana. Malar J. 2013 Jan 17;12:22. doi: 10.1186/1475-2875-12-22, PMID 23327681.
66. Legrand E, Volney B, Lavergne A, Tournegros C, Florent L, Accrombessi D, Guillotte M, Mercereau-Puijalon O, Esterre P. Molecular analysis of two local falciparum malaria outbreaks on the French Guiana coast confirms the msp 1 B-K1/varD genotype association with severe malaria. Malar J. 2005 Jun 21;4:26. doi: 10.1186/1475-2875-4-26, PMID 15969766.
67. Aspeling-Jones H, Conway DJ. An expanded global inventory of allelic variation in the most extremely polymorphic region of *Plasmodium falciparum* merozoite surface protein 1 provided by short read sequence data. Malar J. 2018 Oct 1;17(1):345. doi: 10.1186/s12936-018-2475-2, PMID 30285849.

PICTORIAL ABSTRACT



SUMMARY

The 21st century has witnessed an increase in global travel, and this has reflected in cases of malaria parasite of different species of *Plasmodium* being imported into non-endemic regions as well as those where there is no active transmission. The study looked at genetic diversity of merozoites proteins 1 and 2 of *Plasmodium falciparum* gene. This investigation has shown the genetic diversity in stains of imported case of *P. falciparum* in the region of this study. Encountered genes reflected those seen in different tropical regions of the world. Hence if the area of this study is to achieve a malaria free status, there will be need for continuous monitoring.

Cite this article: Badger-Emeka LI, Al Sultan AA, Al Qatari AA, Al Rashed AS, Al Sayyah A. Molecular Characterization of Genetic Diversity of *Plasmodium falciparum* Associated with Imported Cases of Malaria in Southeastern Region of Saudi Arabia. Indian J of Pharmaceutical Education and Research. 2021;55(4):1183-96.