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# GENETIC DIVERSITY IN THE MSP-1 AND MSP-2 ALLELES AMONG *PLASMODIUM FALCIPARUM* FIELD ISOLATES FROM JAZAN, SAUDI ARABIA

**By**

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**Abstract**

The genetic diversity of *Plasmodium falciparum* infections in human is associated with the pathogenesis of malaria. It is commonly determined through amplification of the polymorphic regions of the merozoite surface proteins -1 (*msp-1*) and -2 (*msp-2*) genes. This study aimed to (1) determine the prevalence of the *msp-1* and *msp-2* allelic familiesand (2) identify the multiplicity of infection (MOI) in *P. falciparum* field isolates from the Jazan region in the Kingdom of Saudi Arabia (KSA).

Blood samples from patients with microscopically confirmed malaria infections (N = 48), collected in 2010, were analysed for *msp-1* and *msp-2* polymorphisms.K1, MAD20 and RO33 allelic types of the msp-1 gene and 3D7 and FC27 alleles of the msp-2 gene were analysed via nested polymerase chain reaction (PCR) according to band size. The MOI was then calculated. In *msp-1*, 16 different alleles were identified by examining size differences in the agarose gels. These alleles-representing 5, 5 & 6 alleles-belong to K1 (120bp-420bp), RO22 (180bp-420bp) and MAD 20 (150 bp-410bp), respectively. For *msp-2*, a larger range of amplicon sizes was detected. A total of 13 different alleles were identified: the FC27 family had 6 alleles (380-bp1280bp), while the 3D7 family had 7 alleles (110 bp-1200bp.MOI was 1.81 for *MSP-1* & 2.17 for *MSP-2,* with overall mean MOI of 1.99).

Considerable genetic diversity was evident in the *P. falciparum* field isolates from the Jazan region of KSA. This diversity represents an essential step in developing effective measures to prevent malaria in KSA, as well as in assessing vaccines derived from these genes.

**Keywords**: Saudi Arabia, Malaria, genetic diversity, *Plasmodium falciparum,* merozoite surface proteins; Multiplicity of infection, Jazan.

## Introduction

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*. *Plasmodium falciparum* is the most virulent species of human malaria and is responsible for most malaria-related deaths. Annually, 300-500 million cases resulted in 800,000 deaths, mainly in tropical and subtropical areas. The majority of deaths as a result of this disease occur in children under five years old (WHO, 2010).

According to the Ministry of Health in Saudi Arabia, malaria is an endemic condition in certain areas of the country. The areas of higher prevalence are located in Jazan, Aseer and the Eastern Provinces, where *P. falciparum* was the most dominant type of malaria, followed by *P. vivax* (Health Statistical Year Book, 2010). The Kingdom of Saudi Arabia borders geographic areas where malaria has historically been endemic but where successful control measures have eradicated these autochthonous infections e.g. Gulf States as Bahrain, Kuwait and the United Arab Emirates (Abdel-Hameed, 2003). On the other hand, in the south-western region of the country, which borders Yemen and Oman, malaria is still prevalent (Ismaeel *et al*, 2004; Al-Mekhlafi *et al*, 2011). Also, imported malaria cases originating from travellers from malaria-endemic areas, pose a major threat for the possible return of autochthonous transmission (Bin Dajem and Al-Qahtani, 2010).

To control and eventually eradicate malaria, effective strategies such as insecticide-treated bed nets (ITNs) have been used. Likewise, the early parasitological diagnosis and treatment of clinical cases must be in mind (Snow *et al,* 2005). Although, these

encouraging strategies, additional measures are required fully to eliminate the disease. A malaria vaccine was considered an essential step (Schwartz *et al,* 2012); however, the genetic diversity of the parasite was a major factor limiting the successful development of an effective malaria vaccine and thus optimal drug therapy (Schwartz *et al,* 2012).

A number of highly polymorphic genetic markers for *P. falciparum* were characterized and can be used to distinguish individual parasite populations and intra-population diversity. The most widely used markers for genotyping *P. falciparum* are the genes encoding merozoite surface protein 1 (*msp-1*), merozoite surface protein 2 (*msp-2*) and to some extent glutamate-rich protein (*glurp*) (Olasehinde *et al,* 2012), which were proposed for use as vaccine antigens because they initiate the immune response in humans (Holder and Blackman, 1994; Moore *et al,* 2002; Elias *et al,* 2013). The *msp-1* is major surface protein in the surface of the merozoites with size of approximately 190 kDa and plays a crucial role in erythrocyte invasion by the merozoite (Woehlbier *et al,* 2006). This antigen has 17 blocks of sequence with conserved regions in between (Tanabe *et al,*1987). Block 2 is the most polymorphic part of msp-1, and is clustered into three main allelic families, namely, the K1, MDA20, and RO33 types (Takala *et al,* 2002). *msp-2* is smaller than *msp-1*(~28 kDa) but is highly polymorphic (Felger *et al,* 1997). It encodes a glycoprotein with five blocks where the central block is the most polymorphic (Felger *et al,* 1997). The *msp-2* alleles are grouped into two main allelic families: FC27 & 3D7 (Smythe *et al,* 1990).

Screening for genetic diversity of malaria parasite populations isolated from KSA (especially *P. falciparum*) is an important step towards development and/or evaluation of malaria vaccines trials and due to their polymorphic features, *msp-1* and *msp-2* were exploited and screened. This would provide trailblazing information on diversity and multiplicity of infection (MOI; number of genotypes per infection) of *P. falciparum* infections in field isolatescollected for the first time from the Jazan region of KSA.

## Subjects, Materials and Methods

Individuals (n=48), who visited the Malaria Centre in Jazan province (Fig 1) during 2010 with malaria infection, were included in this investigation. This study was approved by the Ethics Committee of King Khalid University, Abha, Saudi Arabia. Several drops of blood from each case were blotted onto three MM Whatman® filter paper (Whatman International Ltd., Maidstone, England). The filter papers were air-dried and transported to the laboratory. One blood drop was used for microscopic examination. The blood films were stained with 10% Giemsa solution for 30 minutes. The stained slides were examined by an experienced laboratory technician using a light microscope to determine the presence of malaria parasites.

DNA Extraction: Genomic DNA was extracted from the blood spots of confirmed malaria infection using a Qiagen blood and tissue kit (Qiagen, Valencia, California, USA).Details of the procedure have been described elsewhere (Bin Dajem and Al-Qahtani, 2010)

Identification: *P. falciparum* were molecularly confirmed by 18S rRNA-based nested PCR method by using genus- and species-specific primers (Johnston *et al,* 2006). The procedure was performed using a Qiagen® Fast Cycling PCR Kit (Valencia, California, USA). Known positive control samples and a negative control (nuclease-freewater) were run with each PCR amplification. The second nested PCR products were analysed via 1.5% agarose gel electrophoresis. Photographs were taken using a gel documentation system (Quantum-Capt, Viber Lourmat). The migration distances and band sizes were calculated. The ladder marker used in this study was (*100 bp DNA ladder)* from Qiagen.

Allelic typing of *P. falciparum msp-1* and *msp-2*: Genomic DNA samples (*P. falcipar-*

*um*) were used for the analysis of the two highly polymorphic regions of *msp-1* (block 2) and *msp-2* (block 3) via nested PCR. The whole procedure was described (Viriyakosol *et al,* 1995; Snounou *et al,* 1999). In the primary reactions, oligonucleotide primers corresponded to the conserved sequences within *msp-1* (block 2) and *msp-2* (block 3). In the nested reactions, the separate primer pairs targeted the respective allelic types of *msp-1* (K1, MAD20 & RO33) and *msp-2* (FC27 & 3D7) for amplification. Numbers and sizes of the resulting amplified products were analysed by using a gel documentation system (Quantum-Capt, Viber Lourmat).

Migration distances and band sizes were calculated. Multiplicity of infection (Zwetyenga *et al,* 1998), MOIs (average number of PCR bands per infected case) were calculated by dividing total number of distinct *msp-1* or *msp-2* genotypes detected by positive samples number for same marker.

## Results

Detection of malaria infections by PCR: The study was carried out on 48patients who had a *P. falciparum* infection confirmed by microscopy. Samples were collected from 10 villages in the Jazan area. Nearly 58% of the cases were from non-Saudi patients; 70% were males and 30% were females. The species-specific nested PCR confirmed that all 48(100%) positive cases were infected with *P. falciparum*.

Allelic diversity of *P. falciparum msp-1* & *msp-2* and *MOI*: A high level of allelic diversity was among *P. falciparum* samples. All of 48 positive cases were successfully tested for the *msp-1*(MAD20, K1 and RO33) and the *msp-2*(3D7/IC1 and FC27) allelic families (Figs. 2 & 3). The *msp-1* and *msp-2* alleles were categorized according to the size of the PCR amplicons (Tab. 2).

In *msp-1*, 16 different alleles were identified by examining size differences in the agarose gel: K1 (5), RO22 (5) and MAD20 (6) allelic families. Amplicons with sizes ranged from 120bp to 420bp for K1, 180bp to 420bp for RO22 and 150 to 410bp for MAD20. Larger amplicons were detected for msp-2 gene. A total of 13 different alleles were identified with the FC27 family having 6 alleles that ranged from 380 bp-1280 bp, and 3D7family having 7alleles that ranged from 110 bp to1200bp. Multiplicity of infection (MOI) was 1.81 for the *msp-1*gene, and 2.17for the *msp-2*gene. Overall mean rate of multiplicity was 1.99.

## Discussion

This study revealed considerable allelic diversity and presents first report on the genetic diversity and multiplicity of *P. falciparum* isolates in the Jazan Region. Many studies that used PCR-based genotyping techniques reported extensive genetic diversity in the field isolates of parasites (Babiker *et al,* 2000) and a high degree of genetic diversity created mechanisms for emergence of drug resistance in the *P. falciparum* field parasites (Mahdy *et al,* 2011).

The predominant allele families in *msp-1* & *msp-2* were MAD20 and 3D7, respectively. The results indicated a predominance of MAD20 family of *msp-1*, which agreed with studies in Burkina Faso (Soulama *et al,* 2009), Iran (Heidari *et al,* 2007), Myanmar (Kang *et al,* 2010), Pakistan (Khatoon *et al,* 2010)) and Thailand (Snounou *et al,* 1999). Studies in Tanzania (Schoepflin *et al,* 2009), Pakistan (Ghanchi *et al,* 2010), the Sudan (Hamid *et al,* 2013) and Yemen (Mahdy *et al,* 2011) opposed the present findings.

On the other hand, the analysis of (block 3) of *msp-2* in this study revealed a higher frequency of the 3D7 allelic family than of the FC27 family. A similar prevalence of this family was found in Papua New Guinea and Tanzania (Schoepflin *et al,* 2009), Colombia (Montoya *et al,* 2003), Myanmar (Kang *et al,* 2010), Pakistan (Ghanchi *et al,* 2010; Khatoon e *et al,* 2010) and Thailand (Snounou *et al,* 1999), but these findings were not comparable to those from studies carried out in local endemic countries such Sudan (Hamid *et al,* 2013) and Yemen (Mahdy *et al,* 2011) where FC27 was the more prevalent allele.

Table 1: Sequences of primers used to amplify merozoite surface protein (msp-1 & msp-2) regions

| Gene | Allele | Primer | Sequences |
|---|---|---|---|
| Round 1 | | | |
| msp-1 | | msp1-C1 | AAG CTT TAG AAG ATG CAG TAT TGA C |
| | | msp1-C2 | ATT CAT TAA TTT CTT CAT ATC CAT C |
| msp-2 | | msp2-C1 | ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA |
| | | msp2-C2 | CTT TGT TAC CAT CGG TAC ATT CTT |
| Round 2 | | | |
| msp-1 | K1 | K1a | GAA ATT ACT ACA AAA GGT GCA AGT G |
| | | K1b | AGA TGA AGT ATT TGA ACG AGG TAA AGT |
| | MAD20 | MAD20a | AAA TGA AGG AAC AAG TGG AAC AGC TGT TAC |
| | | MAD20b | ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC |
| | RO33 | RO33a | TAA AGG ATG GAG CAA ATA CTC AAG TTG TTG |
| | | RO33b | CAT CTG AAG GAT TTG CAG CAC CTG GAG ATC |
| msp-2 | FC27 | FC27a | GCA AAT GAA GGT TCT AAT ACT AAT AG |
| | | FC27b | GCT TTG GGT CCT TCT TCA GTT GAT TC |
| | 3D7 | 3D7a | AGA AGT ATG GCA GAA AGT AAG CCT CCT ACT |
| | | 3D7b | GAT TGT AAT TCG GGG GAT TCA GTT TGT TCG |

Table 2: No. of *P. falciparum* alleles and base pair ranges detected per allelic family in MSP1 & MSP2

| Gene | Allele family | PCR product size(bp) | Alleles detected (#) |
|---|---|---|---|
| MSP1 | MAD20 | 150–410 | 6 |
| | K1 | 120–420 | 5 |
| | RO33 | 180–420 | 5 |
| Total | | | 16 |
| MSP2 | 3D7 | 110–1200 | 7 |
| | FC27 | 380–1280 | 6 |
| Total | | | 13 |

The present study proved that the *msp-2* locus is the more diverse in southern Saudi Arabia. This observations agreed with results reported in Senegal (Konate *et al,* 1999), Tanzania (Smith *et al,* 1999) and Sudan (Babiker *et al,* 1997). Thus, the *msp-2* gene can be considered as the most useful marker for estimating *P. falciparum* genetic diversity (Farnert *et al,* 2001). The allele sizes reported for msp2 found to be between 110 bp to approximately 1200 bp, which agreed with studies carried out in India and in Cambodia (Gupta *et al,* 2014; Gosi *et al,* 2013). The diversity of *P. falciparum* within one host can be attributed to the interaction between several factors, i.e. malaria transmission rate and the individual's exposure to different infective mosquitoes (Bruce *et al,* 2000). The high diversity in this study might also be attributed to the mass movement of peoples from Somalia, Eritrea and Ethiopia to Yemen and Saudi Arabia, as a result of political instability, as well as the huge migratory workers from Asia (Soucy, 2011), since these states are endemic malarial areas with a high prevalence of drug-resistant in *P. falciparum*s. Besides, this region annually receives millions of religious pilgrims each year. This immigration increases imported malaria cases, including resistant strains to commonly prescribed drugs within Saudi Arabia (Bin Dajem and Al-Qahtani, 2010).

In meso-endemic areas, mixed infection rates with more than one clone is about 50% and up to100% in in holo-endemic transmission zones (Smith *et al,* 1999). In low endemic area mixed malaria infections can range from 20-30% (Babiker *et al,* 1997).

The MOI was high in this study resembles nearby countries (Yemen, Mahdy *et al,* 2011 & Sudan, Hamid *et al,* 2013), where complexity was similar in hyperendemic areas as in Papua New Guinea (Schoepflin *et al,* 2009), Thailand (Snounou *et al,* 1999) and Pakistan (Ghanchi *et al,* 2010).

## Conclusion

This study represented the first work to suggest utilization of the molecular techniques for exploring the genetic diversity of *P. falciparum* in the KSA.

The future vaccine candidate work may use the depending sequences from these isolates on In order to study in more detail the molecular genetics of the *Plasmodium falciparum* population, extra studies also should include different markers for diversity, such as the inclusion of microsatellites.

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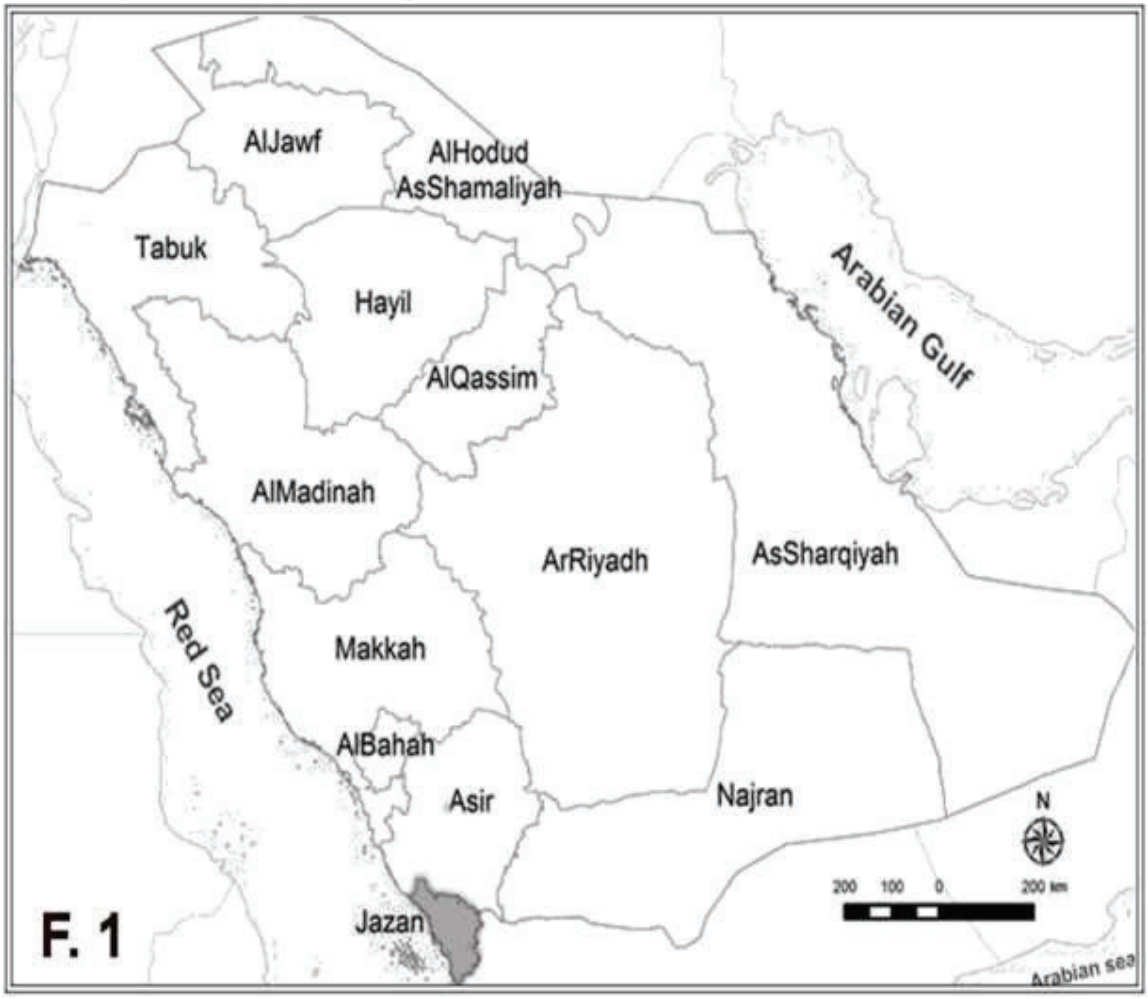


Fig. 1: Map of KSA (http://www.infoplease.com/atlas/country/saudiarabia.html)

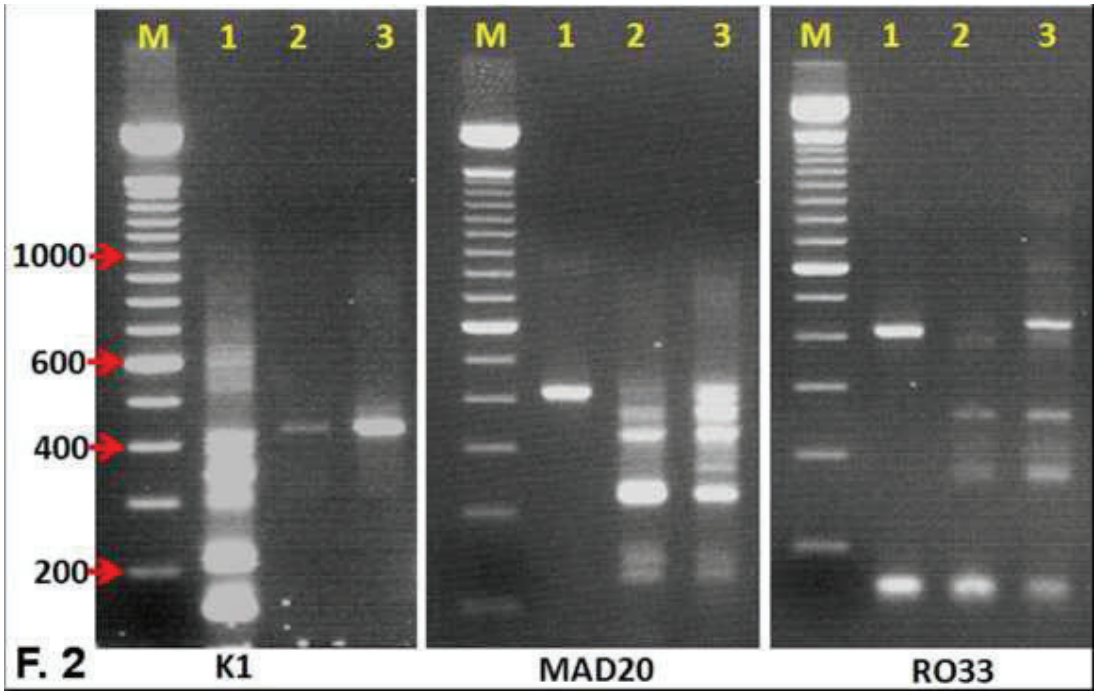

Fig. 2: Agarose gel electrophoresis of PCR products of *msp-1* allelic families (K1, MAD20 & RO33). Lane M: 100 bp DNA ladder plus (Qiagen); lanes 1, 2 and 3: representative samples.

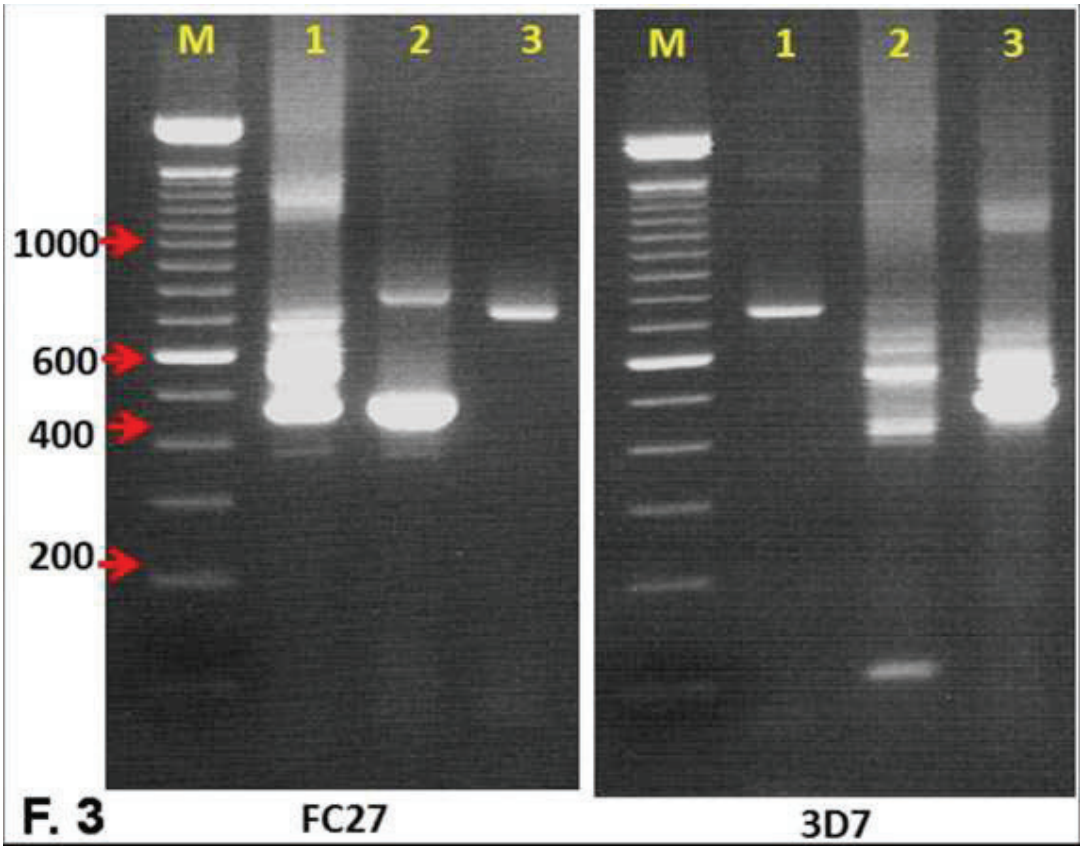

Fig. 3: Agarose gel electrophoresis of PCR products of *msp-2* allelic families (3D7 & FC27). Lane M: 100 bp DNA ladder plus (Qiagen); lanes 1, 2 and 3: representative samples.