Distribution of \textit{msp1}, \textit{msp2} and \textit{eba175} Allelic Family According to Hemoglobin Genotype and G6PD Type from Children with Uncomplicated Malaria in Banfora Heath District (Burkina Faso)

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\textbf{Authors’ contributions}

This work was carried out in collaboration among all authors. Authors SS, IS, YI, AD, AST, ABT, SBS and MMD designed the study, wrote the protocol, performed the study analysis and wrote the first draft of the manuscript. Authors SSS, AD, SAC, AB and DK ensure the participant follow up and collected field data. All authors read and approved the final manuscript.

\textbf{Article Information}

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\textbf{ABSTRACT}

\textbf{Aim:} The present study aimed to evaluate the \textit{Plasmodium falciparum} genetic diversity according to the host hemoglobin and G6PD genetic variants during the course of malaria in infected children aged from 2 to 10 years and living in endemic area in Burkina Faso.

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Study Design: The study was designed as a longitudinal follow up conducted between May 2015 and February 2016 in Banfora health district, Burkina Faso.

Methodology: We included 136 subjects (73 males and 63 females; age range from 2-10 years). Blood thick and thin film was done by capillary blood. Venous blood was collected for DNA extraction. Malaria diagnosis was done by microscopy. Human and parasite DNA were extracted based on Qiagen kit procedure. Then, hemoglobin and G6PD were genotyped by RFLP-PCR while the msp1, msp2 and eba175 genes were typed by a nested PCR. All PCR products were analyzed by electrophoresis on a 1.5-2% agarose gel and alleles categorized according to the molecular weight.

Results: The prevalence of hemoglobin type was 19.11% for abnormal hemoglobin and 80.9% for normal hemoglobin carriage. The prevalence of G6PD type was 91.18% for normal and 8.82% for G6PD deficiency carriage, respectively. The prevalence of msp1 allelic families was 81.60%, 80.80% and 67.20% for k1, ro33 and mad20 respectively while for msp2 gene, fc27 and 3D7 allelic family the prevalence was 70.53% and 69.64% respectively. The eba175 allelic families’ distribution showed 77.31% and 40.21% for fcr3 and Camp respectively. There was no difference in multiplicity of infection (MOI) according to hemoglobin genotypes and G6PD types. We found that k1 was the predominant allelic family of msp1 in normal hemoglobin genotype (AA) and normal G6PD type. The mixed infection of eba175 was statistically higher in abnormal hemoglobin (p=0.04). There was no statistical difference between fcr3 and camp prevalence excepted in G6PD deficient type. The polymorphism results showed that the prevalence of 450 bp in fc27 was statistically significantly higher in normal hemoglobin variant carriers (AA) than abnormal hemoglobin carriers (p=2.10^{-4}). However, the prevalence of 350 bp in fc27 was statistically higher in normal G6PD than deficient G6PD carriers (p=0.034).

Conclusion: Our result showed that the distribution of msp1 and eba75 polymorphism could be influenced by hemoglobin and G6PD variants. These results suggest that hemoglobin and G6PD could influence P. falciparum genetic diversity.

Keywords: Plasmodium falciparum; hemoglobin; G6PD; msp1; msp2; eba175; Burkina Faso.

1. INTRODUCTION

Despite sciences evolution and many efforts by World Health Organization (WHO) and its partners, malaria remains a public health problem in the world. In 2018, an estimated 228 million of malaria occurred worldwide (95% confidence interval [CI]: 206–258 million), compared with 251 million cases in 2010 (95% CI: 231–278 million) and 231 million cases in 2017 (95% CI: 211–259 million) [1]. Most malaria cases in 2018 were in the World Health Organization (WHO) African Region (213 million or 93%), followed by the WHO South-East Asia Region with 3.4% of the cases and the WHO Eastern Mediterranean Region with 2.1% [1]. One of the issues in malaria vaccine discovery is the complexity of the disease. Indeed, malaria is a multifactorial disease modulated by human, parasite, vectors and environmental factors [2]. Previous studies showed that some of the human factor such as abnormal hemoglobin genotype and glucose 6 phosphate dehydrogenase (G6PD) deficiency are potentially protect against severe form of malaria [3,4,5].

Plasmodium falciparum, the most virulent malaria parasite, is known to be very polymorphic. It exhibits a complex genetic polymorphism which may explain its ability to present with different clinical manifestations of the disease spectrum [6]. The relationship between the parasite genotype and the clinical presentation of malaria has already been reported elsewhere [7,8,9].

Moreover, some have stated that certain P. falciparum genotypes can be linked with more virulent infections [10,11]. The presence of multiple infections may affect the release of different pro-inflammatory cytokines as well as making it more difficult for the immune system to deal with infection, resulting in severe malaria [12]. It is also demonstrated that multiplicity of infection did have statistically significant relationship with severity of malaria [13].

As a consequence, some polymorphic genes such as the polymorphic regions of the block 2 of merozoite surface protein-1 (msp-1), block 3 of merozoite surface protein-2 (msp-2) and the RII repeated region of the glutamic rich protein (glurp) or Erythrocytes binding antigen-175 (eba175) are well described as markers for genetic diversity studies of P. falciparum [14]. These different genes are displaying different major allelic families: three major allelic families
have been identified in block 2 of the msp-1 gene, k1, mad20, and ro33 [15], two allelic families in the msp-2 gene, IC/3D7 and fc27 and two for eba175 camp and fcr3 [16]. Erythrocytes binding antigen-175 (eba175), is merozoite surface antigens thought to play a key role in red blood cell invasion by the parasite [17]. These allelic families are used to investigate the genetic diversity, multiplicity of infection, the level of malaria transmission, and to discriminate new from recrudescent infections in therapeutic efficacy monitoring studies [18]. In addition, eba175 C-genotypes were found to be associated with fatal outcome in severe malaria [19]. The characterization of P. falciparum genetic variation according to hemoglobin and G6PD types could help to understand parasite and human interaction during malaria infection which could improve the strategies for malaria elimination. This study aimed to assess the influence of hemoglobin types and G6PD deficiency on the P. falciparum msp1, msp2 and eba175 polymorphism during the course of uncomplicated malaria in a pediatric cohort living in Burkina Faso.

2. METHODOLOGY

2.1 Study Sites

The study was conducted in two villages (Nafona and Bounouna) located in the Banfora Health District, at about 441 km west of Ouagadougou, the capital city of Burkina Faso, in the province of Comoé. The entire health district of Banfora is organized into 24 communities' clinics. Malaria transmission is also markedly seasonal and is intense during the rainy season (May - November). The cumulative annual entomological inoculation rate varies from 55 to 400 infective bites/person/year [20]. P. falciparum is the main parasite present in more than 90% of infections. The incidence rate of uncomplicated malaria (fever and parasitemia 5000/μL or more) in less than five-year-old children is estimated at 1.18 episodes/child-year at risk and around 60% of the total annual number of malaria episodes occurs during the high malaria transmission season [21].

2.2 Patients, Study Design and Sampling

This study was a part of a longitudinal study which involved 150 children aged between 2 and 10 years, males and females who did not showed any symptoms of malaria infection at the time of recruitment. The design of the study consisted of a longitudinal follow up and sampling of children four times for a period of up to 10 months. All children were from two closed-by villages, Bounouna and Nafona. Children were recruited at the end of the dry season with no P. falciparum infection excluding any children with chronic disease or showing clinical signs of other infections. The first sample (Visit 1) was collected before malaria infection followed by weekly follow up where body temperature was taken and the malaria rapid diagnostic test (MRDT) was done. All participants with a positive MRDT and no symptoms (no fever) were referred to CNRFP clinical research unit in Banfora for collection of the second blood sample (Visit 2). Participants do not receive malaria treatment at this stage according to the national malaria treatment guideline but were invited to visit the clinical research unit as soon as an episode of malaria (fever) occurs. The third sample (Visit 3) was collected when an episode of malaria infection (fever with positive MRDT) occurred during the weekly visit or when a participant came passively at the community clinic. In addition to the MDRT, a thick blood smear was performed for participants with a positive MRDT and with a body temperature >37.5°C. Venous blood was also collected at each time visit in EDTA tube for, hematology, G6PD and hemoglobin typing.

Antimalarial treatment was provided following the national guidelines for malaria treatment. Finally, the last blood sample (Visit 4) was collected three weeks after the malaria treatment was initiated.

2.3 Parasite Diagnosis

Blood films were air-dried, thin films fixed with methanol, and the slides were stained with Giemsa 6%. Slides were read following internal standard operation procedure as following: 100 high power fields (HPF) were examined, and the number of malaria parasites of each species and stage recorded. The number of parasites per microliter of blood was calculated, 200 white blood cells per high power field and a fixed white cell count of 8000 /μL. A slide was considered negative if no parasites were found after 100 HPF were examined. Each slide was read by two independent readers. In the event of a discrepancy between the two readers, in terms of species, presence or absence of malaria parasites, or if parasite densities differed by more than 30%, the slide was re-examined by a third laboratory technician. Arithmetic mean of the two
closest readings was used as the final value for parasite density. If there was no agreement after the third reading, the arithmetic mean of the two closest parasite densities was used.

*P. falciparum* infection is defined as a detection of at least one trophozoite on the microscopy field while the clinical malaria was defined as a presence of *P. falciparum* trophozoites associated to an axillary temperature ≥ 37.5°C.

### 2.4 Molecular Analyses

Parasite and human DNA was extracted from blood using QiAmp DNA blood mini kit according to the manufacturer’s protocol (Qiagen; Valencia, CA). Hemoglobin and G6PD genetic variants were typed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). For hemoglobin, DNA samples were amplified using a 5'-AGG AGG GAG GGC AGG A-3' forward primer and a 5'-TCC AAG GGT AGA CCA CCA GTGGCTGTTCCGGGATGGCCTTCTG 3' reverse primer. The PCR conditions used to amplify the three fragments were: 96°C/5 min, 30 cycles of 96°C/30 s, 60°C/1 min, and 72°C/30 s, and final extension of 72°C/5 min. The 358-bases pair (bp) fragment obtained was digested with MnlI (New England Biolabs) and DdeI (Desulfovibrio desulfuricans). The first digestion with the endonuclease MnlI cuts the 5’…CCTC…3’ sequence. It distinguishes AA homozygotes from the AC / AS, SS / SC / CC groups, but not the AS hemoglobin types and the CC, SS HCs. Next digestion with the endonuclease DdeI cuts the 5’C TNAG3’ sequence. It distinguishes between AC and AS on the one hand and SS, SC and CC on the other hand.

For the 202 G>A transition (G6PDA– variant) detection, we used 5' GTCGCTGTTCGGGATGGCCTTCTG 3' forward primer and 5'CTTGAAGAGGGCTCACCTGTGGT 3' reverse primer followed by restriction with NlaIII (New England Biolabs). The PCR conditions used to amplify the three fragments were: 94°C/5 min, 35 cycles of 94°C/30 s, 62°C/30 s, and 72°C/30 s, and final extension of 72°C/5 min for regions including mutations 202 G>A (109 bp product). The 109-base pair (bp) fragment obtained was digested with MnlI for discrimination of mutant type (63 pb, 46 pb). Digestion was carried out for three hours at 37°C and the products were run on a 2% agarose gel [22].

The parasite genetic diversity was assessed during visit 3 based on a nested PCR amplification of *msp1, msp2* and *eba175* gene of *Plasmodium falciparum*. In summary, the primary reaction used a set of primers corresponding to the conserved regions of block 2 for *msp1*, block 3 for *msp2* and region 3 for *eba175*. The second reaction primer set targets specific allelic families of *msp1* (mad20, K1 and r033), *msp2* (3d7 and fc27) or *eba175* (Fcr3 and Camp). Reactions for each set of primary and nested primers were performed separately. A template-free control was used in all reactions and genomic DNA from the American Type Culture Collection was used as a positive control for respective alleles. Cycling conditions for both *msp1*, *msp2* and *eba175* as well as primer sequences are summarized in Table 1 in annex.

All PCR products were analyzed by electrophoresis migration on a 15% agarose gel. DNA fragments were stained with redsafe and visualized by UV transillumination. The sizes of the amplicons were detected using a 100 bp DNA ladder (SD BIOLINE). All the alleles of *msp1, msp2* and *eba175* were categorized according to their molecular weights.

### 2.5 Data Management and Analyses

The data were double entered in a database using an Access 2007 program and analyzed using the software Stata IC version 11.1. Pearson χ² tests and Fisher's exact test were used for comparison of proportions. Student and ANOVA tests were used for comparison of means. *P value* of 0.05 was selected as the threshold of significance for the different statistical tests.

### 3. RESULTS AND DISCUSSION

#### 3.1 Results

##### 3.1.1 Baseline characteristics of study population

Overall, 150 subjects were enrolled in this study. During the follow up, 136 subjects presented *P. falciparum* infection and were included in the genetic diversity assessment. Briefly, the study included 73 male and 63 female subjects. The mean age was 51.37 months (48.35-54.38). The prevalence of hemoglobin type was 19.12% for normal hemoglobin and 80.9% for normal hemoglobin carriage. The prevalence of G6PD type was 91.18% for normal G6PD and 8.82%...
for the deficiency carriage. The mean of parasite density was estimated at 54737 tf/ul while the mean of hemoglobin rate was 10.4 g/l at V3.

### 3.1.2 Prevalence of msp1, msp2 and eba175 allelic families

Out of 136 individual positives for \( P. falciparum \) in microscopy at visit 3, 125 were successfully amplified for msp1 (91.9%), 112 (82.35%) for msp2 and 97 (71.32%) for eba75. In msp1, the k1 and r033 allelic families were predominant with the same prevalence (Fig. 1). We noted a high prevalence of polyclonal infection and the prevalence of subjects harboring together the K1, mad20 and r033 allelic families was up to 50% of polyclonal infection (Fig. 1). In msp2 gene, the 3d7 and fc27 allelic families were around the same prevalence. Monoclonal infection prevalence was more prevalent than the polyclonal infection (Fig. 2). In eba175, fc3 allelic family was most frequently detected compared to camp allelic (\( P=2.10^{-4} \)). The prevalence of subject carrying both genotype fc3 and camp was less than 20%.

### 3.1.3 Polymorphism of msp1, msp2 and eba175 allelic families and hemoglobin and G6PD types

The analysis of the prevalence of the allelic families in each hemoglobin genotype and each G6PD type showed that, the K1 allelic family of msp1 was statistically predominant in normal hemoglobin genotype \( (p=0.01) \) and in normal G6PD type \( (p=0.02) \) (Fig. 1). While the prevalence of the two msp2 allelic families (fc27, 3d7) and the two allelic family of eba-175 (fc3 and camp) were comparable between hemoglobin genotype as well as between G6PD genotype (Fig. 2). The mixed infection prevalence was similar in each hemoglobin genotype and in each G6PD type for msp1 and msp2 while for eba175, the prevalence of mixed infection (fc3+camp) was statistically high in abnormal hemoglobin genotypes than normal hemoglobin genotypes \( (p=0.03) \) (Fig. 3).

### 3.1.4 Genetic diversity and allelic frequency

For both msp1 and msp2, alleles were classified according to the size of the amplified PCR fragment. Fifteen different alleles were observed in msp1 gene with 5 different alleles for k1 (fragment range 150–250 bp), 9 alleles for mad20 (fragment range 100–450 bp) and only one allele for r033 (fragment 150 bp). Among the msp1 fragments, the k1 200 bp, 250 bp and 180 bp; the mad20 200 bp and 180 bp were the most represented. With regard to msp2 gene, 14 individuals distinct alleles were detected with 7 alleles belonging to fc27 family (fragment range 280–700 bp) and 7 alleles to 3d7 family (fragment range 200–700 bp). The 400 bp allele was the most prevalent for fc27 while the 280bp allele was the most prevalent for 3d7. The statistical comparison of the msp1, and msp2 alleles distribution according to hemoglobin genotypes and G6PD type, didn’t confirmed any significant difference in general. However, the 450 bp fragment of fc27 was statistically more represented in normal hemoglobin carriers than abnormal hemoglobin carriers \( (p=2.10^{-4}) \). In addition, the 350 bp of fc27 was statistically predominant in normal G6PD compared to G6PD deficiency carriers \( (p=0.034) \).

![Fig. 1. msp1 allelic families prevalence according to hemoglobin genotype and G6PD type](image-url)
Fig. 2. *msp2* allelic families prevalence according to hemoglobin genotype and G6PD type

![msp2 Allelic Families](image)

Fig. 3. *eba175* allelic families prevalence according to hemoglobin genotype and G6PD type

![eba175 Allelic Families](image)

Fig. 4. K1 fragment according to hb and G6PD

![K1 Fragment](image)
Fig. 5. Mad20 fragment according to hb and G6PD

Fig. 6. 3D7 fragment according to hb and G6PD

Fig. 7. FC27 fragment according to hb and G6PD
3.1.5 Multiplicity of infection (MOI)

In general, based on the diversity, the multiplicity of infection was established as 2.25 and 1.36 in msp1 and in msp2 gene respectively). There was no statistical difference in the MOI according to hemoglobin and G6PD type (p>0.05). The MOI of msp1 is 2.52 for normal hemoglobin, 2.11 for abnormal hemoglobin, 2.42 for normal G6PD and 2.56 for deficient G6PD. For msp2, the MOI was 1.77 for normal hemoglobin, and 1.57 for abnormal hemoglobin, 1.75 for normal G6PD and 1.63 for deficient G6PD.

3.2 Discussion

This study was undertaken to assess the influence of hemoglobin genotype and G6PD type on Plasmodium falciparum genetic diversity. All allelic family of msp1, msp2 and eba175 were presents in our study population.

Plasmodium falciparum genetic diversity depends on many factors like geography, malaria transmission level. In our population, k1 and ro33 was most prevalent and harbored the same prevalence for msp1 allelic families. In a previous study in Bobo Dioulasso where climatic factors and malaria transmission are the same as in Banfora, it was also shown that k1 was predominant. Similar results were shown [23] in Libreville (Gabon) where the k1, was predominant followed by ro33 and Mad20 allelic families. The same results trends were found in isolates from Southwest Ethiopia [16], in Côte d’Ivoire and Gabon [24]. In contrast, some studies found that Mad20 was the most prevalent allelic family [25], Malaysia [26] and Sudan [13].

Allele typing of msp-2 showed that the frequency of fc27 and IC/3d7 allelic families was nearly identical among the isolates; similar to findings from a study in Cambodia [27]. In agreement with previous reports [14,28] and also those reported in a previous studies [29,30,31]. Our results were in contrast with Somé et al. finding in Bobo Dioulasso [32]. They showed that 3d7 prevalence was higher than fc27 prevalence. This difference could be explained by the parasitemia level. In Somé et al study, all subject parasitemia was up to 2000 trophozoite per microliter [32] however our study includes just positive P. falciparum subject. Variations in the prevalence of block 2 alleles between different studies likely reflect differences in geographic locations and local transmission intensity [24,33], in a gene that is highly polymorphic and has a dynamic genetic structure that can reflect transmission pressures [34].

When we compared the distribution of the allelic families in each hemoglobin genotype and in each G6PD type, we found that in msp1, the K1 allelic family was statistically predominant in normal hemoglobin genotype and in normal G6PD type. It looks like in normal hemoglobin and normal G6PD erythrocyte offer best fitness conditions of k1 allelic stains than the others msp1 allelic families. It is known that abnormal hemoglobin genotype and deficient G6PD type protect against severe forms of malaria. The high prevalence of K1 allelic family in normal hemoglobin genotype and in deficient G6PD type suggests that the K1 allelic family of msp1 could affect the severity of malaria. In a previous study in India from severe malaria subject, it also found the predominance of k1 allelic family in normal hemoglobin carrier [35].

There was not statistically difference in msp2 allelic families’ distribution in each hemoglobin genotype and in each G6PD type. That suggests that there was no influence of human genetics factors on msp2 alleles distribution. For eba175 we found that the fc3 allelic family was statistically prevalent in each hemoglobin genotype and in normal hemoglobin type but in deficient G6PD type there was not statistically difference in the prevalence of fc3 and Camp allelic families. In a previous study, Sombié et al. found that the G6PD deficiency mechanism to protect against clinical malaria [36]. In a previous study in Burkina Faso, [37] it was found that that the polymorphism in eba-175 varied between asymptomatic and symptomatic clinical groups and may contribute to the pathogenesis of malaria. Our finding suggests that regulating the eba175 allelic distribution could be on way of G6PD deficiency mechanism to protect against clinical malaria. We found that the prevalence of mixed infection of eba175 was statistically higher in normal hemoglobin genotype than normal hemoglobin genotype. In a previous study, it was found that in the case-control analysis, mixed infections were significantly more common in controls (asymptomatic) than in cases (severe malaria) [19]. This is in accordance with findings from other areas with high transmission [38-39] and is considered to indicate acquired immunity [40]. This could suggest that abnormal hemoglobin protect against severe malaria through immune mechanism. This mechanism could influence eba175 allelic families’ distribution.
There was no statistically significant difference in MOI according to hemoglobin genotype and G6PD type. Our result was in line with the report of Konaté et al. in Senegal. It is known that the MOI is influenced by parasite density Patel et al. [35-41]. In this study, there was not statistically difference on parasite density according to hemoglobin genotype and G6PD type [36]. That could explain our results. However, Patel et al. [35] found a significantly low MOI in abnormal hemoglobin carrier with low parasitemia in abnormal hemoglobin carriers.

4. CONCLUSION

There was no difference in MOI according to hemoglobin genotype and G6PD type in this study but we found that k1 was the predominant allelic family of msp1 in normal hemoglobin genotypes and normal G6PD types. We also noted that the mixed infection of eba175 was statistically higher in abnormal hemoglobin genotype and only in deficient G6PD type the fcr3 of eba175 was not statistically predominant. These results suggest that hemoglobin and G6PD could influence P. falciparum genetic diversity.

CONSENT

Written informed consent was obtained from the parents or legal guardians of all participating children prior to enrolment. In case of any health concerns, the study participant was treated free of charge according to the local standards of care.

ETHICAL APPROVAL

This study was approved by the Ethical Committee of the Ministry of Health of Burkina Faso (Ministry of Health, Burkina Faso; protocol number 2015-02-018) and the Institutional Review Board of New York University Abu Dhabi (UAE, protocol number 011-2015).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


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# ANNEX

## Table 1. Primers sequence and amplification of *msp1*, *msp2* and *eba175*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers sequence</th>
<th>Amplification programme</th>
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<tr>
<td><strong>msp-1</strong></td>
<td></td>
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<tr>
<td>Msp1 A</td>
<td>AAG CTT TAG AAG ATG CAG TAT TGA C</td>
<td>94° 30s</td>
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<tr>
<td>Msp1 B</td>
<td>ATT CAT TAA TTT CTT CAT ATC CAT C</td>
<td>55° 1mn</td>
</tr>
<tr>
<td>K1A</td>
<td>AAG AAA TTA CTA CAA AAG GTG CAA GTG</td>
<td>94° 30s</td>
</tr>
<tr>
<td>K1B</td>
<td>AGA TGA AGT ATT TGA ACG AGG TAA AGT G</td>
<td>62° 1mn</td>
</tr>
<tr>
<td>Ro33 A</td>
<td>AGG ATT TGC AGC ACC TGG AGA TCT</td>
<td>94° 30s</td>
</tr>
<tr>
<td>Ro33 B</td>
<td>GAG CAA ATA CTC AAG TTG TTG CAA AGC</td>
<td>62° 1mn</td>
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<tr>
<td>Mad20A</td>
<td>TGA ATT ATC TGA AGG ATT TGT ACG TCT TGA</td>
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<tr>
<td>Mad20B</td>
<td>GAA CAA GTC GAA CAG CTG TTA</td>
<td>58° 1mn</td>
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<tr>
<td><strong>msp-2</strong></td>
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<td>MSP2 4</td>
<td>ATA TGG CAA AAG ATA AAA CAA GTG TTG CTG</td>
<td>55° 1mn</td>
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<td>FC27 A</td>
<td>GCA AAT GAA GGT TCT</td>
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<td>FC27 B</td>
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<td>3D7 A</td>
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<td>3D7 B</td>
<td>GAT TTG TTT CGG CAT TAT TAT GA</td>
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<td>3D7 B</td>
<td>GAT TTG TTT CGG CAT TAT TAT GA</td>
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<td><strong>eba-175</strong></td>
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<td>eba2</td>
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<td><strong>eba-175</strong></td>
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<td>CAA TTC CTC CAG ACT GTT GAA CAT</td>
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