

Weak D in the Tunisian population

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Background. More than 90 weak D types have been discovered to date. As there are no published data on the frequencies of weak D types in the Tunisian population, the aim of this study was to determine the composition of weak D alleles in our population.

Materials and methods. Blood samples from 1777 D+ and 223 D- blood donors were tested for markers 809G, 1154C, 8G, 602G, 667G, 446A, and 885T relative to translation start codon by polymerase chain reaction with sequence-specific primers to estimate the frequencies of weak D type 1, weak D type 2, weak D type 3, weak D type 4, weak D type 5 and weak D type 11 in our population. Twenty-three samples with positive reactions were re-evaluated by DNA sequencing of RHD exons 1-10 and adjacent intronic sequences.

Results. Among the D+ donor cohort, weak D type 4 was the most prevalent allele (n=33, 1.2%) followed by weak D type 2 (n=6, 0.17%), weak D type 1 (n=4, 0.11%), and weak D type 5 (n=1, 0.28%) and weak D type 11 (n=1, 0.28%). *RHD* sequencing identified a weak D type 4.0 allele in all 19 samples tested. Among the D- pool, comprising 223 samples, we detected one sample with weak D type 4.0 associated with a C+c+E-e+ phenotype which had been missed by routine serological methods.

Discussion. Weak D type 4.0 appears to be the most prevalent weak D in our population. However, all samples must be sequenced in order to determine the exact subtype of weak D type 4, since weak D type 4.2 has considerable clinical importance, being associated with anti-D alloimmunisation. One case of weak D type 4 associated with dCe in *trans* had been missed by serology, so quality control of serological tests should be developed in our country.

Keywords: RH system, weak D, PCR-SSP, sequencing, Tunisia.

Introduction

In the field of blood transfusion, correct determination of the D phenotype is of pivotal importance in avoiding the risk of alloimmunisation. One of the most significant developments in the past decade has been the identification of a considerable number of *RHD* alleles that encode for partial and weak D phenotypes^{1,2}.

Partial D types are the result of *RHD/RHCE* gene hybridisation events or point mutations in *RHD* stretches encoding extracellular RhD domains with characteristic loss of D epitopes. Weak D expression results primarily from single nucleotide polymorphisms in the *RHD* gene that encode amino acid substitutions predicted to be in the intracellular or transmembrane regions of RhD polypeptides¹. These single nucleotide polymorphisms affect the efficiency of insertion and, therefore, the quantity of RhD polypeptide in the erythrocytes, leading to a reduced number of D antigen sites on the red blood cells.

Newly discovered *RHD* alleles may be considered for classification as numerically dubbed weak D types if they fulfil two main characteristics:

(i) haemagglutination using standard serological methods is observed to be weaker than that observed for regular RhD; (ii) the observed *RHD* sequence contains missense mutations in comparison to the *RHD* wild-type sequence, encoding amino acid substitutions in transmembrane or intracellular parts of the RhD protein^{1,3}.

Weak D types types 1, 2 and 3 are not associated with the development of alloantibodies; however, alloimmunisation has been reported in carriers of weak types 4.2, 11, 15 and 21^{1,2-4,6}.

There is substantial ethnic variability in partial D and weak D types. Some alleles are confined to specific ethnic groups whereas others are more widely distributed. The most prevalent weak D types in Europeans are type 1, type 2 and type 3, which account for 93.49% of all weak D types⁷. Other aberrant *RHD* alleles are more frequent in African populations (weak D type 4.2, DIVa, DAU-4, and DOL)⁸⁻¹⁰.

The current Tunisian population has an ancient Berber background and has been influenced by the different civilisations that settled in this region in historical times: Phoenicians (814-146 B.C.,

Romans (146 B.C.-439 A.D.), Vandals (439-534 A.D.), and Byzantines (534-647 A.D.). By the end of the 7th century A.D., migrants from the Arabian Peninsula had expanded the Islamic religion and Arabic language into North Africa. Between the 16th and 19th centuries, various waves of immigration by Turks, Spanish and French had also occurred. The Berber people presently live in several areas of North Africa (Morocco, Algeria, Tunisia and Libya), as well as some Saharan regions in Mauritania, Niger, Chad, Mali and Senegal. Approximately 1% of the global Tunisian population is still distinctly Berber; these people are mainly localised in the south of Tunisia^{11,12}.

Numerous studies on several red blood cell markers have confirmed the mixed roots of the Tunisian population^{13,14}. In this work we performed a systematic study at the molecular level of 2,000 blood donors to determine the composition of weak D alleles in our population. We have been able to establish the population frequency of the weak D variants that are known to be most prevalent in Europeans (weak D types 1-3, 5 and 11) as well as in Africans (especially weak D type 4). Our findings have also given some insight into the genetic variability of our population and have important implications in transfusion medicine, allowing better management of alloimmunisation and improving transfusion outcomes.

Materials and methods

Blood samples from random blood donors in Tunisia were collected into ethylene-diaminetetraacetate (EDTA) anticoagulant between October 2010 and September 2011. Two blood transfusion centres participated in this study: the *Centre Régional de Transfusion Sanguine* (CRTS) of Sousse (1,000 samples: Sahel of Tunisia) and the *Centre National de Transfusion Sanguine* (CNTS) of Tunis capital (1,000 samples: north and south of Tunisia).

Serological testing

Serological testing involved haemagglutination with two commercially available monoclonal antibody reagents from Diagast (Loos, France) and Biomaghreb (Tunis, Tunisia) containing a blend of both IgG and IgM anti-D. Diagast and Biomaghreb reagents were used to test for anti-D (RH1, clones P3×61+P3×21223B10+P3×290+P3×35). These clones agglutinate with several partial D, in particular the D category VI (DVI).

Bio-Rad reagents (Marnes-la Coquette, France) were used to test the following specificities: anti-C (RH2, clone MS24), anti-E (RH3, clone MS260), anti-c (RH4, clone MS33) and anti-e (RH5, clones MS16, MS21, MS63). Red blood cell phenotyping was performed according to the manufacturer's

instructions. Samples that tested negative with anti-D in the direct agglutination tests were retested using indirect agglutination tests.

Molecular analysis and RHD genotyping

DNA was isolated by a modified salting-out procedure following the technology described by Miller *et al.*¹⁵, and quantified by optical density measurement with Nanodrop® 1000 (Nanodrop Technologies, Wilmington, DE, USA).

Screening by polymerase chain reaction with sequence-specific primers

Polymerase chain reaction with sequence-specific primers (PCR-SSP) was applied, using previously reported conditions^{16,17}, to detect the 809 T>G, 1154 G>C, 8 C>G, 602 C>G, 667T>G, 446 C>A and 885 G>T polymorphisms associated with weak D type 1, weak D type 2, weak D type 3, weak D type 4, weak D type 5, and weak D type 11 alleles, respectively. All D samples (D+ and D-) were also tested with primers specific for the wild-type *RHD* gene targeting exons 4 (602 C), 5 (667 T) and 6 (809 T) (Table I).

To avoid false-negative results, an internal control amplifying a 429 bp segment of the human growth hormone gene was included in each reaction¹⁸. Amplifications were carried out with Taq Invitrogen (Invitrogen, São Paulo, SP, Brazil) in a final volume of 10 µL. The reactions worked under similar PCR conditions on a DNA thermocycler (9700 GeneAmp PCR System; Applied Biosystems, Foster City, CA, USA). Cycling conditions consisted of an initial denaturation of 2 minutes at 94 °C, followed by ten cycles of 10 seconds denaturation at 94 °C and 1 minute annealing/extension at 65 °C, and finally 22 cycles of denaturation for 30 seconds at 94 °C, 1 minute annealing at 61 °C and 30 seconds of extension at 72 °C. After amplification, 5 µL of the final PCR products were analysed on 2% agarose gels.

RHD zygosity determination by real-time quantitative polymerase chain reaction

To determine *RHD* zygosity, a real-time quantitative PCR was employed (Model 7700 sequence detector, Applied Biosystems) under conditions described previously¹⁹.

DNA sequencing

RHD exons 1 to 10 including intron/exon border areas were investigated for some *RHD* alleles (19 weak D type 4, 1 weak D type 1, 1 weak D type 2, 1 weak D type 5, and 1 weak D type 11) by nucleotide-sequencing using BigDye chemistry, v.3.1 (Applied Biosystems) and a genetic analyser (ABI 3100,

Table I - Primers for the PCR-SSP detection of weak D types.

RHD exon	Nucleotide exchange	Primer name	Primer direction	Nucleotide sequence	Specificity	Position ^d
Exon 6	809 T>G	gwd1a ^a	Forward	5'acacgctatttcttgacACTTATG T 3'	<i>weak D type 1</i>	-19-809
		gwd1a ^b	Forward	5'acacgctatttcttgacACTTATG G 3'		-19-809
		gwd1b ^a	Reverse	3'GGTACTTGGCTCCCCCGAC5'		934-916
Exon 9	1154 G>C	gwd2a2 ^a	Forward	5'ctccaaatctttaacattaaattgcatcctaaacag C 3'	<i>weak D type 2</i>	-38-1154
		rg94 ^a	Reverse	3'cctcctgcaatgctccttactc5'		142-120
Exon 1	8 C>G	gwd3a ^a	Forward	5'acagagacggacacaggATGAAT G 3'	<i>weak D type 3</i>	-17-8
		gwd3b ^a	Reverse	3'CTTGATAGGATGCCACGAGCC5'		148-127
Exon 4	602 C>G	gwd4 ^a	Forward	5'AGACTACCACATGAACATGATGCACA3'	<i>weak D type 4 (T201R)</i>	489-514
		gwd4b ^a	Reverse	3'CAGACAAACTGGGTATCGTTGCT G 3'		625-602
		gwd4b ^b	Reverse	3'CAGACAAACTGGGTATCGTTGCT C 3'		625-602
Exon 5	667 T>G	Rh223v ^f ^a	Forward	5'TTGTGGATGTTCTGGCCAAGT T 3'	<i>weak D type 4 (F223V)</i>	646-667
		Rh223v ^f ^b	Forward	5'TTGTGGATGTTCTGGCCAAGT G 3'		646-667
		ga51 ^a	Reverse	3'CTGCTCACCTTGCTGATCTTCCC5'		787-758
Exon 3	446 C>A	gwd5aneu ^a	Forward	5'GGTGCTGGTGGAGGTGACGG A 3'	<i>weak D type 5</i>	446-466
		gwd5 ^a	Reverse	3'gagcttggccctttctccc5'		51-29
Exon 6	885 G>T	ga62 ^a	Forward	5'TTGGCCATGCACAGTGGGTGTTGG3'	<i>weak D type 11</i>	804-826
		Rh2591b ^a	Reverse	3'cagccacaagaccagcaca 5 '		904-885

^aSpecific for wild-type alleles; ^bFrom (Müller *et al.*, 2001)⁷; ^c(Wagner *et al.*, 2001)¹⁷; ^dThe positions of the synthetic oligonucleotides are indicated relative to their distances from the first nucleotide position of the start codon ATG for all primers in the promoter and in the exons, or relative to their adjacent exon/intron boundaries for all other primers. PCR-SSP: polymerase chain reaction with sequence-specific primers.

Applied Biosystems) as described previously²⁰⁻²³. Data were analysed using SeqScape v. 2.7 (Applied Biosystems).

Results

Population survey

The RH phenotype of all 2,000 blood donors was determined by routine serological methods. We identified 1,777 D+ samples (88.85%), and 223 D- samples (11.15%). The phenotype frequencies are summarised in Table II.

Among the D- pool, comprising 223 samples, the results of PCR-SSP for both wild-type and mutated alleles showed the presence of one sample with weak D type 4 (weak D type 4/weak D type 4 or weak D type 4/deletion) (Figure 1) which had been missed by the routine serological typing.

Out of 1,777 samples with D+ phenotypic characteristics, there were 45 positive amplifications by PCR-SSP: 4 samples (0.11%) were positive for the weak D type 1-specific marker 809G, 6 (0.17%) for the weak D type 2-specific marker 1154 T, 33 (1.2%) for the weak D type 4-specific markers 602G and 667G and one each (0.028%) for the weak D type 5-specific marker 446 A and for the weak D type 11-specific marker 885 T.

Among the 34 subjects with weak D type 4 (33 D+ and 1 D-) detected in our cohort, only 11 individuals were found to harbour the weak D type 4 markers (602G and 667G) in a homozygous state (10 samples typed D+C-c+E-e+ and 1 sample D-C+c+E-e+) (Figure 1).

Table II - Results of serological Rhesus typing.

Phenotypes	Number of samples	Frequencies (%)
D+C+c+E-e+	728	36.4
D+C+c-E-e+	422	21.1
D+C-c+E-e+	230	11.5
D+C-c+E-e+	182	9.1
D+C+c+E-e+	182	9.1
D+C-c+E-e-	26	1.3
D+C+c-E-e+	7	0.35
dd C-c+E-e+	198	9.9
dd C+c+E-e+	19	0.95
dd C-c+E-e+	3	0.15
dd C-c+E-e-	2	0.1
dd C+c-E-e+	1	0.05
Total	2,000	100

Homozygosity of the weak D type 4 allele was determined by PCR-SSP (absence of amplification of *RHD* exons with wild-type primers) and confirmed by real-time quantitative PCR for five samples tested. Hence, the phenotype frequency of weak D type 4 was 0.55% and its allele frequency was determined as 1.2%.

Full-length nucleotide sequencing of *RHD* exons 1-10 identified a weak D type 4.0 allele in all 19 samples tested (4 in a homozygous state and 15 in a heterozygous state). The former four samples (1 weak D type 1, 1 weak D type 2, 1 weak D type 5, and 1 weak D type 11) were also confirmed by sequencing. The RhCE phenotyping of these variants is presented in Table III.

As regards the weak D alleles found in this study, weak D type 4 was the most common, occurring with a frequency of 73.91%, followed by type 2 (13.04%), type 1 (8.69%) and both type 5 (2.17%) and type 11 (2.17%) (Figure 2).

Discussion

In this systematic population investigation of random blood donors from Tunisia, we identified a total of 45 weak D alleles among 1,777 D+ samples and one weak D type 4 among 223 D- samples. Type 4 was the most prevalent weak D type allele (1.2%), followed by type 2 (0.17%), type 1 (0.11%) and types 5 and 11 (0.028% each). Of the 46 weak D alleles found in this study, 34 were weak D type 4 alleles and in 11 cases the markers 602G and 667G, specific for weak D type 4, were present in a homozygous state. *RHD* zygosity by real-time quantitative PCR confirmed the presence of two copies of the *RHD* gene in five individuals tested.

The weak D type 4.0 allele harbours 602 C>G and 667 T>G substitutions located in *RHD* exons 4 and 5. It is, however, now known that there are several "suballelic" variants (4.0, 4.0.1, 4.1, 4.2.1, 4.2.2, 4.2.3 and 4.3)⁴. In our cohort, sequence analysis of *RHD*

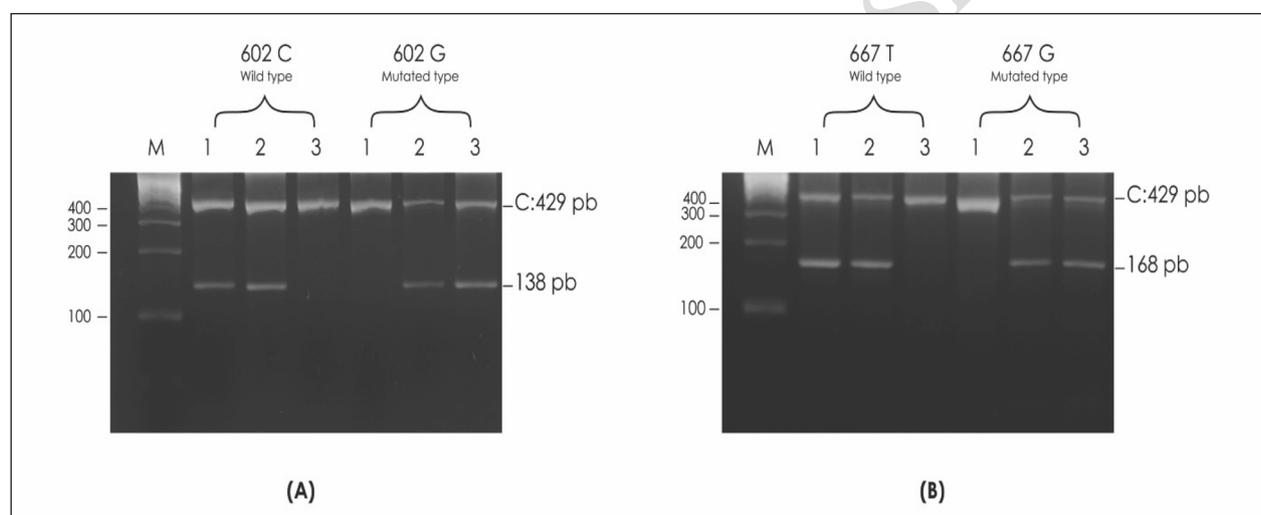


Figure 1 - Results of two PCR-SSP specific for weak D type 4.

(A) Results of PCR-SSP specific for the 602 C>G substitution (RHD exon 4).

(B) Results of PCR-SSP specific for the 667 T>G substitution (RHD exon 5). M: size marker; C: internal control 1: D+ sample (RHD/RHD or RHD/deletion) 2: Weak D type 4 in a heterozygous state (weak D type 4/ RHD) 3: Weak D type 4 (weak D type 4/weak D type 4 or weak D type 4/deletion). PCR-SSP: polymerase chain reaction with sequence-specific primers.

Table III - Weak D alleles and their associated phenotypes.

weak D category	PCR-ASP nt	Number of samples	Phenotypes
weak D type 1	809T/G	4	C+c+E-e+
weak D type 2	1154 G/T	6	C-c+E+e+
weak D type 4	602G/G 667G/G	10	C-c+E-e+
	602G/G 667G/G	1	C+c+E-e+ (typed D-: missed by routine serology)
	602C/G 667T/G	23	C-c+E-e+ (n=4) ; C+c+E+e+ (n=1); C+c+E-e+ (n=17), C-c+E+e+ (n=1)
weak D type 5	446 C/A	1	C-c+E+e+
weak D type 11	885G/T	1	C-c+E-e+
Total		46	

PCR-ASP: polymerase chain reaction with allele-specific primers.

exons 1-10 was performed for only 19 of the 34 weak D type 4 alleles (33 typed D+ and one typed D-). A weak D type 4.0 allele was identified in all these individuals. Hence, weak D type 4.0 seems to be the most prevalent weak D in our population. It does, therefore, appear to be important that this variant should be detected by serology and typed as D+.

The four samples tested which were found to harbour the markers specific for weak D type 1, weak D type 2, weak D type 5, and weak D type 11 phenotypes were also confirmed by sequencing. We cannot, however, exclude the possibility that other unknown subtypes of weak D type 4 and type 1 may have been present, because we did not sequence all our samples.

The frequency of weak D type 4 allele found in our population (1.2%) was higher than that in white people but lower than that in black Africans. In fact the cumulative frequency of weak D type 4 was 17.2% in South African blacks¹⁰ and 0.0055% in white blood donors¹.

Among the weak D alleles found in this study, type 4 was the most common, with a frequency of 73.91%, followed by type 2 (13.04%), type 1 (8.69%) and types 5 and 11 (2.17% each) (Figure 2). As expected, when compared to European data, showing that weak D types 1-3 are most frequent⁷, and African data, showing that weak D type 4 is very frequent¹⁰, our data demonstrate the presence of the common signs of these two ancestries and confirm the mixed origin of our population.

Weak D types and the associated phenotypes found in this study were consistent with those described in other studies. It has been reported that weak D type 1 is associated with the C+c+E-e+ phenotype. Weak D type 2 is the most frequent cause of weak D among C-c+E+e+ individuals¹. The weak D type 4 allele is associated with the ce allele of the *RHCE* gene, whereas weak D type 5 and weak D type 11 have been associated with the cE and ce allele of the *RHCE* gene respectively¹.

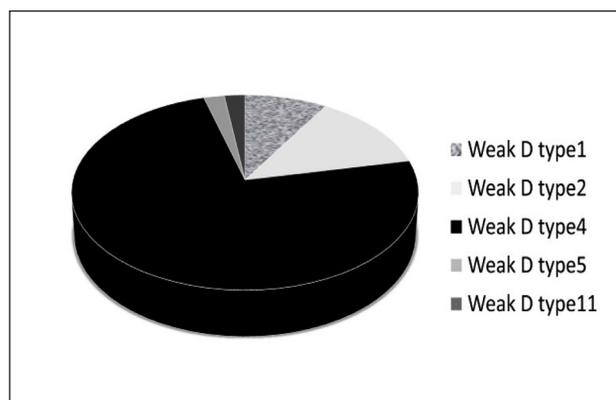


Figure 2 - Composition of weak D alleles in the Tunisian population.

The published frequencies of several weak D types in European countries differ, which may give rise to serological problems and carry a distinct potential for alloimmunisation. It is well established that individuals with weak D type 1, type 2 and type 3 do not produce alloanti-D²⁴⁻²⁸ and rarely autoanti-D²⁹. It is, therefore, a reasonable policy to treat patients with those variants as D+ , in order to conserve stocks of D- red cells, and to treat those with any other variant as D-⁴. This will not adversely affect D- stocks as all of the other variants are relatively rare. In Croatia, the use of *RHD* gene molecular genotyping in cases of ambiguous serological interpretation of D antigen allowed for the use of D+ red blood cell units in 87.4% of cases (weak D types 1-3)³⁰. In Denmark, the use of *RHD* gene molecular genotyping was estimated to reduce the use of D- blood by 3%³¹.

It is important to know the exact subtype of all weak D type 4. Indeed, the management of weak D type 4 differs depending on the subtype. Pregnant women and recipients of blood transfusions expressing the weak D type 4.2 variant should be regarded as D- and require anti-D prophylaxis¹⁰.

Weak D type 4.0 appears to be the most prevalent weak D in our population; however, no serological means for its identification was available. It has been reported that weak D type 4.0 is associated with low-titre anti-D which precludes auto- and allo-anti-D from being distinguished³². Although the frequency of weak D type 4 genotype (weak D type 4.0/weak D type 4.0 or weak D type 4.0/deletion) in Tunisia is 0.55%, we have reported only one case of anti-D auto-immunisation in poly-transfused patients with weak D type 4.0³³. According to Flegel's recommendations, patients with weak D type 4.0 may be transfused with D+ red blood cells³².

In our study, we found a very low frequency of weak D type 5 and type 11 alleles. Weak D type 5 and type 11 have an antigen density of 296 and 183, respectively¹. Hence, they are rarely typed as D+ by routine serology. Indeed, in a previous study¹⁴ a weak D type 11 was omitted by serology and typed RH:-1. To prevent the risk of alloimmunisation it is recommended that D- red blood cells are transfused into patients with these phenotypes. Although weak D donations may be mistaken for D- and transfused to D- patients, red cells with very weak D phenotypes do not appear to be very immunogenic³⁴.

Among 223 D- samples, we identified one weak D type 4 which had been missed by routine serology. Serological tests for this variant certainly need to be further evaluated; however, in our laboratory, we have detected another case of weak D type 4 among D- samples¹⁴, which was typed as D+ by the indirect agglutination test.

This variant was associated with the C+c+E-e+ phenotype. The presence of the dCe haplotype in *trans* may decrease the antigen density through the Ceppellini effect³⁵. Weak D type 4 associated with C antigen was previously described in D- Tunisian individuals¹⁴ and in D- Congolese blood donors (>6%)³⁶. Since weak D type 4.0 with dCe in *trans* was missed by serology in our laboratory and yet it is the most prevalent variant in Tunisia, quality control of serological typing of such red blood cells is recommended.

All D- samples were analysed by wild-type primers specific for three *RHD* gene fragments including exons 4, 5, and 6. The absence of amplification with these primers confirmed that the deletion is the most prevalent mechanism for D- in the Tunisian population, as reported in a previous study³⁷.

In our study, we used PCR-SSP to determine the composition of weak D types that were known to be more prevalent in Europeans as well as in Africans. Screening the *RHD* gene in D- samples is very important for the detection of *RHD* variants. Since *RHD* exon 10 is conserved in all *RHD* variants other than DHar, targeting this exon is very sensitive for *RHD* gene detection in D- samples. In a previous study¹⁴, *RHD* genotyping in D- blood donors using a PCR specific for *RHD* exon 10 allowed us to identify the presence of two cases of weak partial 11, and one each of weak partial 4.0, weak D type 29 and one DBT-1 partial D.

Conclusion

In conclusion, we analysed a large number of samples and determined the frequency and composition of weak D alleles in our population. These data will help us to implement the best alloimmunisation anti-D prevention strategy in our population.

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Authorship contributions

OM, RH, CT, AS, HB, HS, and J-YS performed the research study. OM, J-YS and RH analysed the data. J-YS and HS contributed essential reagents and tools. OM and J-YS wrote the paper.

The Authors declare no conflicts of interest.

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