

# Mutations causing hemophilia B in Algeria: Identification of two novel mutations of the factor 9 gene

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**Abstract.** *Abla Z, Mouloud Y, Hejer El, Emna G, Abdi Meriem A, Ouarhlent Yamina O, Naouel S. 2018. Mutations causing hemophilia B in Algeria: Identification of two novel mutations of the factor 9 gene. Biodiversitas 19: 52-58.* Hemophilia B (HB) (also known as Christmas disease; Christmas is the family name of the first patient.) is an X linked recessive hemorrhagic disorder caused by mutations in factor 9 (*F9*: is used for the gene) gene that leads to deficient or defective coagulation factor IX (FIX: is used for the protein). The variable phenotype of HB results from wide range of mutations affecting the *F9* gene. Our study was aimed at molecular analysis of HB to identify the causative mutation in known patients with HB in a part of Algeria. For genotyping, polymerase chain reaction (PCR) and direct sequencing have been applied to all the essential regions of the *F9* gene from 39 Algerian HB patients belonging to 13 unrelated families. We identified 10 different mutations. The identified mutations included 1 duplication and 9 substitutions. In total 9 point mutations were identified, of which 5 are located in exon 8, the hotspot region in the *F9* gene. Among the 10 mutations, 2 are novel and not deposited in database sites nor described in recently published articles. The results of this study emphasize the heterogeneity of HB. In summary, our preliminary results will be used to build an Algerian mutation database which would facilitate genetic counseling.

**Keywords:** Hemophilia B, *F9* gene, Mutations, Molecular analysis.

## INTRODUCTION

HB is a sex-linked genetic disorder resulting in a deficiency of plasma coagulant activities of FIX (Lillicrap 1998). This factor is located in the long arm of the X chromosome, in q27,1. The *F9* gene contains eight exons and seven introns (Anson et al. 1984; Yoshitake et al. 1985). The exons code for the FIX protein, which is a vitamin K-dependent serine protease, it is present in plasma as a glycoprotein of 415 amino acids, activated by FVIIa and FXIa (Roberts 1993). This factor is divided into six domains: prepropeptide, the Gla domain, epidermal growth factor EGF1 and EGF2 domains, the activation domain, and the catalytic domain.

HB is due to a variety of mutations distributed over the entire *F9* gene, and described in patients affected by HB of varying severity (Pei-Chin et al. 2014). Since the cloning of the *F9* gene in 1982, its analysis in different patients has helped to identify many different genetic abnormalities leading to hemophilia. Among them, we distinguish major anomalies (large deletions, mutations that lead to a stop codon: nonsense), source of a lack of transcription and therefore of an absence of synthesis of FIX. Other mutations can lead to the expression of a non-functional FIX (missense point mutations, small insertions or deletions causing splice abnormalities) (Chambost and Meunier 2006).

The main objective of this study was, to identify the mutations that produce different forms of HB disease among Algerian patients, to characterize mutations of the *F9* gene and to reinforce our understanding of the molecular basis of this disease.

## MATERIALS AND METHODS

### Patients

39 patients with HB from 13 unrelated families of Algerian origin were included in this study. At the time of the study, their age ranged between 4 to 84 years. 26 patients with severe form, 9 with moderate form and 4 had mild form of HB. Informed consent was obtained from each family for molecular studies.

### Blood collection and hematological laboratory analysis

The blood sample of the study subjects was taken from venipuncture into evacuated tubes containing trisodium citrate (0,109 M) as an anticoagulant. The activity of FIX (FIX: C) is measured using partially activated thromboplastin. The principle of the assay consists in determining, in the presence of cephalin and activator, the coagulation time where all the factors are present, except FIX provided by the plasma of the patient to be tested. Severity is classified based on the amount of FIX activity,

severe if < 1%, moderate if between 1 and 5% and mild if > 5 and < 40% of normal. Clotting factor inhibitor screening was performed by activated partial thromboplastin time mixing studies using normal pool plasma. These blood samples were provided by the hematology service of the University Hospital Center (UHC) of Batna and Constantine, Algeria.

### Molecular genetic analysis

About 5 to 10 ml of peripheral blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA). Deoxyribonucleic acid (DNA); support of our genetic information, was isolated from white blood cells by the salting out method of Miller et al. (1988) and stored at 4°C. Quality and quantity of DNA are estimated by spectrophotometry. All of the eight *F9* exon regions (including flanking sequences), the putative promoter region, and the polyadenylation signal region were amplified by PCR using the primer sequences given in Table 1. Exons 2 and 3 were grouped into a single fragment; exon 8 was divided into 2 fragments for PCR amplification. PCR is a method of gene amplification in vitro, which allows duplicating in large numbers (with a multiplying factor of the order of one billion), a known DNA sequence, from a low quantity (of the order of a few picograms) of nucleic acid.

DNA is the raw material of our study, its quality is essential and conditions the success of our analyzes. Before genotyping, the quality and quantity of DNA are methodically evaluated. To determine if amplification has responded to criteria expected (specificity, efficiency and absence of contamination), the PCR products are checked on an agarose gel whose concentration is a function of the size of the amplicon. The HB mutation was identified by automated sequence analysis performed on an ABI3100

Genetic Analyzer (Applied Biosystems) using the Capillary electrophoresis method. The reading and the comparison of the sequences analyzed, to the consensus sequences, are carried out using the computer software BioEdit. Once the sequencing results are obtained, an analysis of the genomic variations found and a bibliographic search in the different databases is carried out in order to distinguish the mutations reported from those that are newly identified. Mutation nomenclature was given according to Genetic Variations approved by the Human Genome Variation Society (HGVS) (<http://www.hgvs.org>). Results were examined using Ensembl genome browser (<https://www.ensembl.org/index.html>).

To analyze the nature of the novel missense mutation and to predict the possible impact of an amino acid substitution on the structure and function of FIX, we used PolyPhen-2 (Polymorphism Phenotyping-2) (<http://genetics.bwh.harvard.edu/pph2>), I-Mutant 2.0 (<http://folding.biofold.org/cgi-bin/i-mutant2.0.cgi>), Align GVGD (Align Grantham Variation Grantham Deviation) (<http://agvgd.iarc.fr/>) and Project HOPE (<http://www.cmbi.ru.nl/hope/>).

## RESULTS AND DISCUSSION

In this study, 39 HB patients from 13 families were investigated. Screening for molecular events in the FIX gene in these 13 families led to the characterization of 9 point mutations, of which one was novel; missense mutation in exon 8, and 1 novel duplication of four nucleotides in exon 6. No mutation was detected in two families. The causative mutation within FIX was identified in 85% of families. Mutations found in Algerian HB were shown in Table 2.

**Table 1.** Primers for mutation analysis of *F9* gene.

Functional region	Sequence of the primer	Product size (base pair «bp» )
Promoter	5'-CAAGCTACAGGCTGGAGACA-3' 5'-TCTCCCTCAATGGGTCTTTG-3'	410
Exon 1	5'-TTCAGACTCAAATCAGCCACA-3' 5'-AAAAGGCAAGCATACTCAATGT-3'	354
Exons 2-3	5'-CAAAGACTTTCTTAAGAGATGT-3' 5'-GACAAAGTTTAATATATTATCTAT-3'	557
Exon 4	5'-ATCCCAATGAGTATCTACAGG-3' 5'-CACCAATATTGCATTTTCCAG-3'	275
Exon 5	5'-ATACATGAGTCAGTAGTTCCA-3' 5'-AGGAAGCAGATTCAAGTAGG-3'	309
Exon 6	5'-TCTCAGAAGTGACAAGGATG-3' 5'-ACATCCCAATAGGTCTGTCT-3'	407
Exon 7	5'-CTATTCCTGTAACCAGCACA-3' 5'-CTTCTGCCTTTAGCCCAATT-3'	318
Exon 8p	5'-TTGCCAATTAGGTCTAGTGGTC-3' 5'-ATGTGGCTCGGTCAACAAGT-3'	400
Exon 8d	5'-TTTGCAATGCTGACAAGGAA-3' 5'-GCCCTGTTAATTTCAATTCCA-3'	436
Poly A	5'-ACTAGCATAACCCCGAAGTG-3' 5'-CGCCGCCGCGACTGATTCACAT-3'	265

**Table 2.** Identified causative mutations in Algerian hemophiliacs B

Family	No. of patients	FIX: C	Severity	cDNA change (HGVS notation)	Amino acid change	Type	Mechanism	Location	Domain	CpG	Inhibitors	Novel/Reported
1	6	<1%	Severe	c.-52C>T	-	Promoter	Substitution	5'UTR	-	-	No	Reported
2	7	<1%	Severe	c.323G>A	p.C108 Y	Missense	Substitution	Exon 4	EGF1	No	No	Reported
3	2	<1%	Severe	c.357T>A	p.C119*	Nonsense	Substitution	Exon 4	EGF1	No	No	Reported
4	4	1%	Moderate	c.373G>A	p.G125R	Missense	Substitution	Exon 4	EGF1	No	No	Reported
5	1	<1%	Severe	c.657_660dup ATCA	p.D223*	Frameshift	Duplication	Exon 6	Activation peptide	-	No	Novel
6	3	3%	Moderate	c.881G>A	p.R294Q	Missense	Substitution	Exon 8	Serine protease	Yes	No	Reported
7	2	<1%	Severe	c.881G>A	p.R294Q	Missense	Substitution	Exon 8	Serine protease	Yes	No	Reported
8	1	<1%	Severe	c.892C>T	p.R298*	Nonsense	Substitution	Exon 8	Serine protease	Yes	No	Reported
9	4	7%	Mild	c.1010C>T	p.A337V	Missense	Substitution	Exon 8	Serine protease	No	No	Reported
10	3	<1%	Severe	c.1150C>T	p.R384*	Nonsense	Substitution	Exon 8	Serine protease	Yes	No	Reported
11	1	<1%	Severe	c.1184T>C	p.F395S	Missense	Substitution	Exon 8	Serine protease	No	No	Novel
12	3	<1%	Severe	-	-	-	-	-	-	-	No	-
13	2	2%	Moderate	-	-	-	-	-	-	-	No	-

Note: FIX: C = factor IX coagulant activity. \* = stop codon

Our study, represent an approach for the molecular diagnosis of HB in our country. Our cohort consists of 39 male patients from 13 unrelated families. The predominance of HB in the male sex is explained by the mode of transmission of the disease. Patients of the same family have the same degree of severity; this is also explained by the mode of transmission of the disease. Guérois (2009) wrote that most of the time, the same type of hemophilia would be found in the same family (A or B) and the same degree of severity, this is usually related to the transmission of the same mutation between several individuals of the same family.

The databases that record HB mutations indicate a total of 1095 mutations corresponding to 3713 cases. According to some studies (Rallapalli et al. 2013; Surin et al. 2016; Morteza et al. 2007; Nazia et al. 2008; Pei-Chin et al. 2014; Morteza et al. 2009; Tengguo et al. 2014), point mutations are the most common alterations in HB, and present in almost 90% of patients, whose largest proportion of patients with HB have missense mutations, which is in agreement with our results, missense mutations were identified for 6 of 11 families.

Genetic defects in exons 4 and 8 which successively encode the EGF-like domain and the serine protease domain are more widespread among the mutations described in patients with HB because of the importance of the amino acids for which they code (Rallapalli et al. 2013). As reported in the literature, the majority of point mutations of *F9* gene are located in exon 8 which is the largest exon of the *F9* gene (Anson et al. 1984; Rallapalli et al. 2013; Tengguo et al. 2013). This is also the case for our patients, most mutations are in exon 8 (5/10), followed by exon 4 (3/10).

### Novel mutations

Out of 13 HB families which DNA samples have screened, we have found 2 novel mutations, one was a missense mutation and the other was a frameshift mutation.

A novel missense mutation p.F395S was detected in one family (family 11). This T>C mutation at residue 1184, occurred in codon TTC of phenylalanine (F) to change into TCC serine (S) and this amino acid is present in exon 8 within the Serine protease domain. F395 is in a disulfide loop formed by Cys382 and Cys396. The replacement of a nonpolar acid by a polar acid can affect the function, secretion or stability of the protein, as is the case for the new mutation p.F395S. Chromatogram of this mutation was shown in Figure 1.

Using straightforward physical and comparative considerations, PolyPhen-2 predicts possible impact of an amino acid substitution on the structure and function of a human protein. According to PolyPhen-2, this mutation is predicted to be probably damaging with a score of 1.000. Using PolyPhen-2, enabled us to obtain the result of multiple alignments compared to all the species recorded on UniProtKB, which enabled us to confirm that this mutation is at a conserved position among several species.

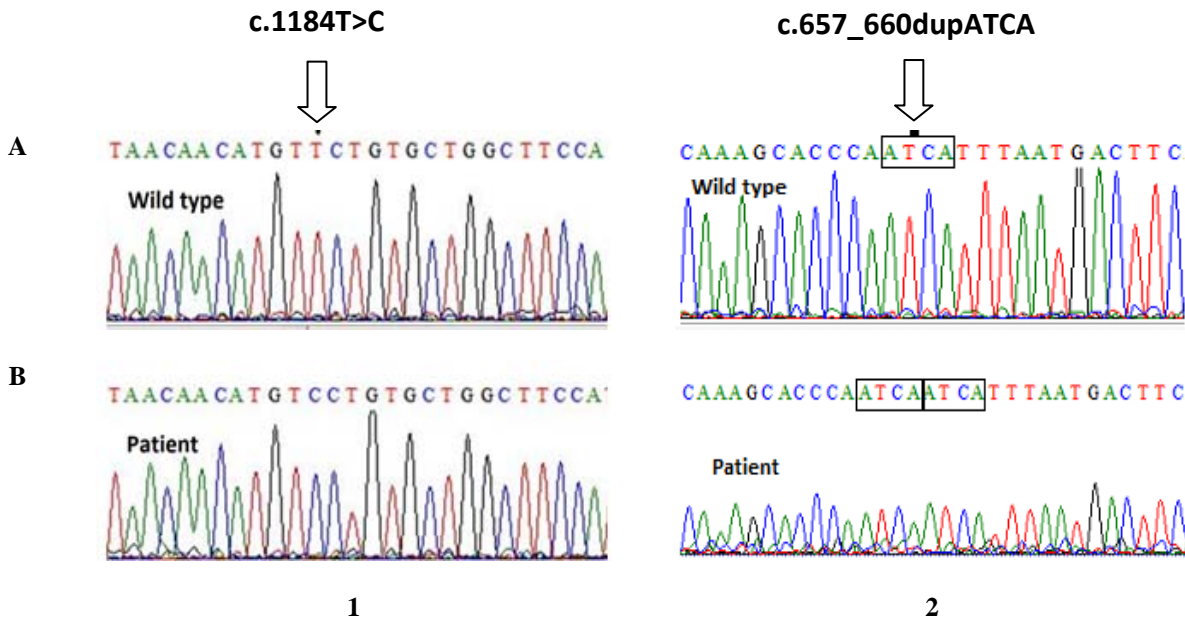
The analysis of the effects of this novel missense mutation on the stability of the FIX protein has been predicted by the I-Mutant 2.0 logiciel. I-Mutant 2.0 was used to calculate the energy difference (DDG) between normal and mutated protein. The calculated DDG value is -2.89. Since this value is negative, the mutation tested was considered destabilizing for the FIX structure.

Align GVGD software has also been used to predict the effects of this mutation on the FIX protein. The analysis of this mutation by Align GVGD made it possible to calculate

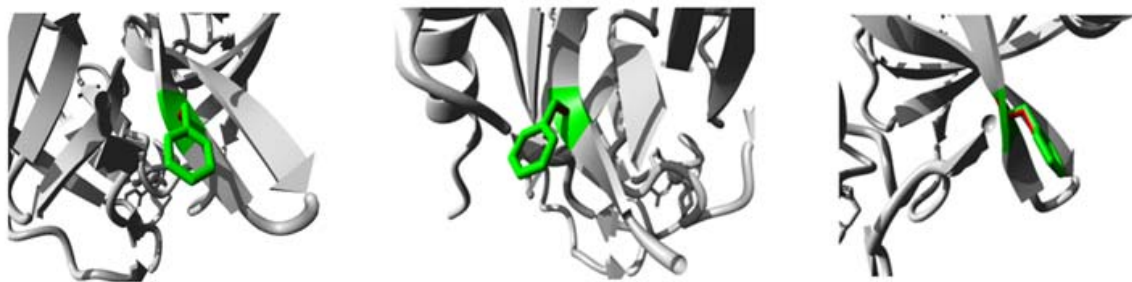
the GV and GD values. The value of the Grantham variation (GV) is 0.00 which means that the substitution sits at the level of a highly conserved region between the species analyzed. On the other hand, this same logiciel allowed us to calculate the Grantham deviation (GD) between wild and mutated amino acid. The GD value for the p.F395S mutation is equal to 154.81 which mean that the replacement of phenylalanine by a serine is predicted as a radical change.

Using Project HOPE software, information from the 3D-structure was obtained using WHAT IF Web services, the UniProt database and the Reprof software. The structural information was obtained from the analysis of PDB using the code 3LC3. The comparison between original wild-type residue (phenylalanine) and newly introduced mutant residue (serine) showed that they differ in several properties. The mutation introduces an amino acid with different properties, which can disturb the serine

protease domain and abolish its function. The wild-type and mutant amino acids differ in size; the mutant residue is smaller than the wild-type residue. The mutation will cause an empty space in the core of the protein. The hydrophobicity of the wild-type and mutant residue differs. The mutation will cause loss of hydrophobic interactions in the core of the protein. In the PDB file used for this analysis, the mutated residue is involved in a multimer contact. The mutation introduces a smaller residue at this position. The new residue might be too small to make multimer contacts. The mutation introduces a less hydrophobic residue. Sometimes, hydrophobicity is important for multimerisation and therefore this mutation could affect the multimer contacts. For this mutation, the two 3D structures of wild and mutated amino acid were superimposed to determine any differences in size and orientation of the side chains (Figure 2).



**Figure 1.** 1. Novel missense mutation identified in exon 8 of the *F9* gene was found in family 11. A. DNA sequence of the normal control. B. DNA sequence of the patient. 2. Novel duplication identified in exon 6 of the *F9* gene was found in family 5. A. DNA sequence of the normal control. B. DNA sequence of the patient



**Figure 2.** Superimposed capture of the two 3D structures of wild and mutated FIX for mutation p.F395S, produced by Project HOPE (view on three different angles). The FIX protein is represented in gray ribbon form. The side chain of the amino acid phenylalanine is shown in green while that of the amino acid serine is shown in red.

Another novel mutation was identified in one severely affected patient (Family 5), it is a frameshift mutation, and it was caused by the novel duplication c.657\_660dupATCA which was due to insertion of four nucleotides (ATCA) in Activation peptide domain encoded by exon 6, leading to a stop codon at position 223 (p.D223\*). The presence of a stop codon at position 223 of the *F9* gene would generate a truncated polypeptide and would eliminate a part of activation peptide domain and all the catalytic domain of the mature FIX protein. Chromatogram of this mutation was shown in Figure 1.

Small insertions located in coding regions are responsible, when the number of inserted nucleotides is different from a multiple of 3, of a frameshift when translating mRNA into protein, which results most often at the appearance of a stop codon. These frameshift or shifting mutations are usually associated with the severe form of the disease (Giannelli and Green 1996; Tuddenham and Cooper 1994; Antonarakis et al. 1995; Rafati M et al. 2011).

### Reported mutations

In our study, one mutation localized in the proximal promoter region was identified. This mutation at position -52 causing the C to T substitution, found in family1, it was previously described in moderate case (Ketterling et al. 1995). However, in our patient cohort, this mutation was associated with a severe disease. According to the study of Lannoy et al. (2017), this c.-52 position is situated over a segment of 6 nucleotides (TGACT) (-56 to -51) that is common to ARE (-65 to -51) site and HNF4 $\alpha$  (-56 to -48) element. Mutations in this position disrupt androgen receptor (AR) and hepatic nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ). Androgen response element (ARE); sequence which binds to testosterone from puberty and activates *F9* transcription, HNF4 $\alpha$  is a binding site which the protein facilitating *F9* transcription.

We identified 4 previously reported missense mutations in 5 unrelated families with HB. Two of them are located in exon 8 (p.R294Q and p.A337V), and two other (p.C108Y and p.G125R) are located in EGF1 domain which is encoded by exon 4.

In our patients, the mutation p.C108Y was associated with severe disease. This mutation was previously found in three patients from United Kingdom, Germany, and USA and the last was in moderate form (Haris et al. 1994; Thorland et al. 1995; Rallapalli et al. 2013). p.G125R was found in one family (family 4) with moderate HB. This mutation was previously published by Epsinos et al. (2003) for a patient with the severe form of the pathology. One mild form was caused by p.A337V in one family (family 9). As our results, two patients with this mutation were reported previously with the mild form (Weinmann et al. 1998; Rallapalli et al. 2013). Our results are in agreement with many studies, which confirm that missense mutations were associated with mild, moderate and severe form (Belvini et al. 2005; Rallapalli et al. 2013; Lannoy et al. 2017).

In the study of Johnsen et al. (2017), Missense mutations accounted for most of the mutations detected in males with mild or moderate HB. The deleterious effect of a missense mutation is based on the importance of the domain where the mutation is located (location of the mutation), the degree of conservation of the amino acid and the nature of the amino acid change. Each amino acid has its own specific size, charge, and hydrophobicity-value. The original wild-type residue and newly introduced mutant residue often differ in these properties. Mutations that introduce an amino acid with different properties, which leads to disrupt the protein and abolish its function.

Severe and moderate forms of HB resulting from (p.R294Q) in two unrelated families, have been observed in our study. Severe, moderate and mild forms of HB resulting from this mutation have been reported earlier (Rallapalli et al. 2013). This finding concurs with the study of Belvini et al. (2005), this mutation and others were found in more than one patient with phenotypically different disease severity. According to Elmahmoudi et al. (2012), the discrepancy may be explained by the intervention of other hemostatic factors which can modify the clinical severity of hemophilia.

Three nonsense mutations were found by us, occurred in three independent families with the severe phenotype, all of them previously listed in CHBMP database. The two mutations (p.R298\*, p.R384\*), present in the serine protease domain; and p.C119\* present in the EGF1 domain. The replacement of cysteine by a stop codon at position 119 has been previously described in a patient with severe form (Ghanem et al. 1993). p.R298\* and p.R384\* were previously described in patients bearing a moderate and severe disease (Rallapalli et al. 2013). Our results are in agreement with the conclusion of Belvini et al. (2005), nonsense mutations are expected to produce truncated unstable proteins regardless of their location and to result in a severe disease, because the existence of a stop codon causes premature stop of translation. And because of the involvement of the Nonsense-Mediated mRNA Decay (NMD) system, these incomplete proteins can neither synthesize nor secrete into the circulation. The NMD is a mechanism for degrading a selective mRNA having a premature stop codon, thereby preventing translation of a truncated protein potentially deleterious. p.C119\* causing a protein lacking the three domains: EGF2, activation peptide, and serine protease. According to Chen et al. (1989), The existence of a stop codon at position 298 of the *F9* gene would generate a polypeptide chain slightly greater than half its normal length (297/461) and eliminate much of the catalytic domain of FIX protein mature. The replacement of arginine by a stop codon (p.R384\*), leading to a protein devoid of part of the catalytic domain (383/461).

Three mutations (p.R294Q, p.R298\* and p.R384\*) were at CpG dinucleotides which are considered to be mutation hot spots, the CpG sequence is a highly susceptible site for mutation of the *F9* gene (Chen et al. 1989). The first mutation involves a G→A, the other mutations are nonsense and involve a C→T. The

mechanism of this phenomenon due to the spontaneous deamination of 5'-methylcytosine to thymidine (T) (Belvini et al. 2005).

The *F9* open reading frame has 20 CpG sites, 6 of which reside in CGA codons. The C to T transition particularly destroys the CGA Arg codon and converts it to the TGA stop codon, which is associated with severe HB (Youssofian et al. 1988; Koeberl et al. 1990).

According to the high number of publications of the mutations p.R294Q and p.R298\* (more than 97 publications for p.R294Q and more than 63 articles for p.R298\*), they are considered to be frequent mutations. This is explained by the poor conservation of arginine at position 294 and position 298 among serine proteases (Greer 1990), would make tolerable most substitutions.

#### **Absence of identification of mutation responsible for HB**

For patients with families 12 and 13 presenting diagnostic criteria of HB: 3 cases with a severe form and two cases with a moderate form, search for a mutation at level of *F9* gene remained negative. This same result was reported in three patients of Indian origin (Mahajan et al. 2004). It is possible that this family might have pathological translocation, duplication or inversion in the *F9* gene leading to the disease. Further investigation is needed. According to Nguyen (2010), direct genetic diagnostic techniques (Denaturing high-performance liquid chromatography (DHPLC), capillary electrophoresis sequencing) can accurately identify the mutation responsible for severe hemophilia for most patients and families. It is estimated that a mutation is detected in 96% of cases of severe hemophilia A and B and 80% of cases of moderate and minor hemophilia A and B.

#### **Inhibitor development**

The occurrence of an inhibitor antibody by alloimmunization remains the major complication of alternative therapy for hemophiliacs. This complication presents 35% of cases of severe hemophilia A and 5% of cases of severe HB according to the latest studies (Gouw et al. 2013; Srivastava et al. 2013; Kessler et al. 2015). Inhibitors are less common in patients with HB. The type of the mutation in the *F9* gene is attached to a genetic predisposition to the appearance of inhibitors. Most occur in patients with large deletions or nonsense mutations (Goodeve 2015). Pinotti et al. (2012) revealed that patients with nonsense mutations display a lower risk of inhibitors than patients with deletions. The studies of (Thorland et al. 1999) and (Warrier and Lusher 1998) showed that patients with deletions of the *F9* gene are at high risk of developing inhibitors. In our cohort, no deletion mutation was identified. In two studies reporting hemophilia from Iran, neither of patients with HB developed inhibitor (Morteza et al. 2007; Mehdizadeh et al. 2009) similar to our study, FIX inhibitors have not been observed in our patients. This was probably due to absence of deletions of the *F9* gene in our cohort or to the small sample size, in order to confirm this results we need to increase the number of the investigated HB patients in our study.

In the present study, we report the molecular analysis of causative mutations in Algerian HB patients. We confirm the genetic heterogeneity of *F9* mutations leading to HB in Algeria. In total, we have identified 10 mutations including 2 novel mutations. These 10 mutations include 3 nonsense mutations (p.C119\*, p.R298\*, p.R384\*), 5 missense mutations (p.C108 Y, p.G125R, p.R294Q, p.A337V, p.F395S), 1 mutation at the promoter (c.-52C>T), and 1 duplication (c.657\_660dupATCA). The novel missense mutation p.F395S is predicted to be probably damaging. The frameshift mutation was caused by the novel duplication c.657\_660dupATCA, leading to codon stop, which causes premature stop of translation. In general, most of the mutations identified are family-specific except the p.R294Q mutation that has been identified in two unrelated families. Identification of these mutations in the *F9* gene has contributed to our understanding of molecular pathology of HB in Algeria. Since the Algerian population is genetically heterogeneous, our study is an initiative to complete the molecular spectrum of hemophilia B patients in our country which will establish a molecular database whose purpose is to improve prenatal diagnosis, genetic counseling, the diagnosis of female carriers in families at risk. They are also valuable in future gene therapy studies.

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