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The Application of Next-Generation Sequencing for Hemophilia A and B Genotyping.

Fanny Lassalle*[1], Antoine Rauch[2], Benedicte Wibaut[1], Christine Vinciguerra[3], Mathilde Fretigny[3], Bertrand Vaast[2], Valerie Gomanne[1], David Lillicrap[4], Sophie Susen[2], Jenny Goudemand[2], Christophe Zawadzki[2]

[1]Department of Hematology and Transfusion, Lille University Hospital, Lille, France, [2]Department of Hematology and Transfusion, Lille University Hospital & Inserm UMR1011, University of Lille, Pasteur Institute, EGID, Lille, France, [3]Department of Hemostasis, Edouard Herriot Hospital, Lyon, France, [4]Department of Pathology and Molecular Medicine, Queen's University, Kingston, Canada

Background: The identification of molecular defects in hemophilia is essential for the optimization of patient treatment and the formal characterization of female carriers. The Sanger method is the gold standard for sequencing the *F8* and *F9* genes but is time-consuming and expensive.

Aims: We aimed to develop a high-throughput method to genotype hemophilia A (HA) and B (HB) patients using the Next-Generation Sequencing (NGS) technology for an exhaustive and less expensive analysis of the *F8* and *F9* genes.

Methods: We developed a small panel containing the *F8* and *F9* genes. We compared the performances of 3 protocols called P1 (AmpliSeq™ library preparation kit on a PGM™ Ion Torrent sequencer), P2 (HaloPlex™ kit on a Proton™ Ion Torrent sequencer) and P3 (HaloPlex™ kit on a MiSeq™ Illumina sequencer). NGS analysis was first performed in 61 samples previously characterized for *F8* or *F9* mutations by Sanger method or Multiplex Ligation-dependent Probe Amplification (MLPA). All types of mutations were studied (nonsense, missense, splicing, small insertion/deletion (indels) and exons deletion/duplication) and were distributed in all exons of *F8* and *F9*. NGS analysis was further performed in 42 hemophilia patients with unknown mutation status. Most of them presented a mild phenotype (36/42). All patients provided a written informed consent. Data were analyzed with SeqNext software (JCI Medical System™). A Normalized Reads Depth (NRD) ratio was used to detect exons deletion/duplication.

Results: The mutations of previously-genotyped patients were detected in 90, 84 and 95% of cases with P1, P2 and P3 respectively. Indels in homopolymers of exon 14 in *F8* were well-detected with P3 but incorrectly with P1 and P2. Copy Number Variants (CNV) were only detected with P1. No false-positive was detected. The sensitivity of the 3 protocols was 90, 84 and 95% respectively with the same specificity of 100%. A mutation was detected in 90% (38/42) of not previously genotyped patients. Eleven new variants were identified, including 9 missense mutations. Of the 7 missense mutations in

F8, 4 were confirmed to be deleterious by *in vitro* mutant expression. No mutation was found in 11% (4/36) of not previously genotyped patients with mild HA, consistent with the available data for this phenotype. We also evaluated the time of labor and the cost of the three NGS protocols in comparison to Sanger reference method. Overall, NGS was 2 to 4-fold faster and less expensive than Sanger sequencing. P3 was the most cost efficient of our three protocols being faster and less expensive than P1 and P2.

Conclusion: NGS is able to detect the main types of mutations in the *F8* and *F9* genes. In our study, indels in homopolymers in *F8* were only well detected with the Illumina technology (MiSeq™, P3) and CNV only detected with the AmpliSeq™ kit (P1). We identified 5 new variants (4 missense and one nonsense) and confirmed their deleterious effects. In our lab, HaloPlex™ chemistry in combination with the MiSeq™ sequencer appears as a competitive solution for an accurate, fast and cost-effective sequencing of *F8* and *F9* genes in hemophilia.