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Utility of Multiplex Ligation-Dependent Probe Amplification (MLPA) for Hemophilia Mutation Screening

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Keywords

F8 mutations; *F9* mutations; Hemophilia; Multiplex Ligation-Dependent Probe Amplification

Hemophilia A (HA) and B (HB) are estimated to affect 1 in 5,000 male births in the United States each year.[1] Inheritance of mutations in the Factor VIII (*F8*) gene or Factor IX (*F9*) gene causes these bleeding disorders. Identification of mutations causing a patient's hemophilia can lead to better understanding of risk of complications [2], as well as aid in carrier detection in family members [3]. Mutation screening for HA has involved testing for inversions of introns 1 and 22 of *F8*, as approximately 45% of severe HA patients carry an inversion as their causative mutation [2], and sequencing of the coding regions of *F8* to identify point mutations, deletions, or splice-site mutations. Similarly, mutation screening for HB has involved sequencing of the coding regions of *F9*. However, a subset of patients presenting with hemophilia do not to have a detectable mutation with these methods.[4] Duplication of part of *F8* or *F9*, for example, may not be detected. Also, female family members heterozygous for a large *F8* or *F9* deletion may not be identified as carriers using these methods, as dosage of the genes is not determined. Recently, Multiplex Ligation-Dependent Probe Amplification (MLPA®, MRC Holland, Amsterdam, Netherlands) has been successfully used to identify large deletions and duplications within *F8* and *F9*.[5-7] This assay quantitatively compares copy numbers of a set of DNA sequences in a patient sample to those in a control sample to screen for the presence of deletions or duplications.[8] The assessment of how this or similar technologies will fit into currently-used mutation screening protocols should be critically evaluated.

We describe here our experience using MLPA® in combination with inversion testing and DNA sequencing for identification of mutations in a large series of hemophilia patients. Additionally, we present our analyses of how this duplication/deletion testing fits into mutation screening algorithms and highlight the importance of careful assay validation and interpretation.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

A subset of patients enrolled in the Hemophilia Inhibitor Research Study were studied for this report.[2] At the time of this study, mutation analysis had been completed on 930 HA patients enrolled in the Hemophilia Inhibitor Research Study (250 more patients than in the previous report) and 152 HB patients. Patients with no mutation detected by inversion testing or sequencing and patients with large deletions detected by sequencing were screened with MLPA® SALSA Kits P178 for *F8* and P207 for *F9* (MRC Holland, Amsterdam, The Netherlands) following the manufacturer's protocol. MLPA® fragments were separated and sized using the 3730 DNA Analyzer (Applied Biosystems, Carlsbad, California, USA) and analyzed using the GeneMapper® 4.0 (Applied Biosystems) and Coffalyzer® (MRC Holland) software packages. Large duplications were confirmed with TaqMan® CNV assays (Applied Biosystems) custom-designed to assay the region of the duplication following the manufacturer's protocol.

Of the 930 HA patients included in the study, 47 (5%) patients who did not have a mutation detected by inversion testing or *F8* sequencing and 28 (3%) HA patients with large deletions within *F8* were screened. Large duplications were identified in 8 (17%) patients with no mutation previously detected and were confirmed with TaqMan® CNV assays. All large deletions initially identified by sequencing were confirmed by MLPA®. Figure 1A outlines the algorithm used to identify mutations in this patient population. MLPA® allowed the identification of 8 more mutations in this population, resulting in 96% of patients having an identified mutation.

Of the 152 HB patients included in the study, 2 (1%) patients with no mutation detected by *F9* sequencing and 6 (4%) patients with large deletions were screened with MLPA®. No large duplications were identified. All large deletions were confirmed by MLPA®. Figure 1B outlines the algorithm used to identify mutations in this patient population. MLPA® did not allow the identification of any more mutations in this population. However, it did allow the confirmation of all of the large deletions detected by sequencing.

In the Hemophilia Inhibitor Research Study 5% of severe HA patients and 13% of severe HB patients carry a large deletion or duplication.[2] Similar distributions have been seen in other population-based studies.[9-11] Inhibitors are estimated to occur in approximately 50% of HA and HB patients with large deletions or duplications.[2, 12] Our experience screening *F8* and *F9* with MLPA® resulted in the successful confirmation of all 28 deletions in *F8* and all 6 deletions in *F9* identified by DNA sequencing. Furthermore, 8 duplications were identified in 47 HA patients with no previous mutation identified.

In order to most efficiently identify mutations and classify those at-risk for developing inhibitors as this technology enters the clinical setting, we propose the screening algorithms outlined in Figure 1. Because approximately 40% of HA patients carry an inversion and because inversion carriers are at moderate risk for inhibitor development [12], we propose initially screening for *F8* inversions in patients with HA. If no inversion is identified, *F8* sequencing would be subsequently conducted. It is estimated this would identify over 95% of mutations leading to HA.[2, 4] If no mutation is identified through sequencing, MLPA® should then be conducted to identify possible large duplications. Similarly, we propose initially sequencing *F9*, followed by MLPA® if no mutation is identified.

In carrier screening for either HA or HB, the algorithm would be altered depending on the mutation identified in the index case. For example, if a point mutation or small insertion/deletion were identified in the index case, only the amplicon for the gene region surrounding the mutation would need to be sequenced to determine carrier status. Because large deletions and duplications in carriers would result in an altered copy number in the region of the deletion or duplication, we propose using MLPA® to identify carriers of these mutations, as sequencing would not be able to detect these copy number changes.

The proper use of MLPA® or similar technologies to screen for genomic deletions or duplications in hemophilia requires careful attention to several key aspects: choice of control samples; interpretation of results; and choice of DNA extraction methods. Because *F8* and *F9* are X-linked genes, it is necessary that reference samples be obtained from subjects of the same sex as the tested patient in order to correctly measure the ratio of copy numbers in the samples. It is also important to consider the assay-specific control probe composition when using male reference samples to assay female carriers or, alternatively, using female reference samples to assay male patients. For example, the *F8* MLPA® control probe sequences are in X-linked genomic regions, while those in the *F9* kit are autosomal. Also, the assay is sensitive to sample impurities and inconsistencies may be introduced by using multiple DNA extraction methods. Finally, due to the complicated nature of quantitative assays to determine copy number variation, assay validation is essential. Initial results must be confirmed by repeated testing with an independent method, such as quantitative PCR.

MLPA® kits surveying the *F8* and *F9* gene are useful for identifying deletions and duplications causing HA and HB. The kits help to identify previously-undetectable mutations and provide a method for rapid identification of patients most at-risk for complications of inhibitors. However, appropriate caution must be taken to validate results and ensure correct interpretation.

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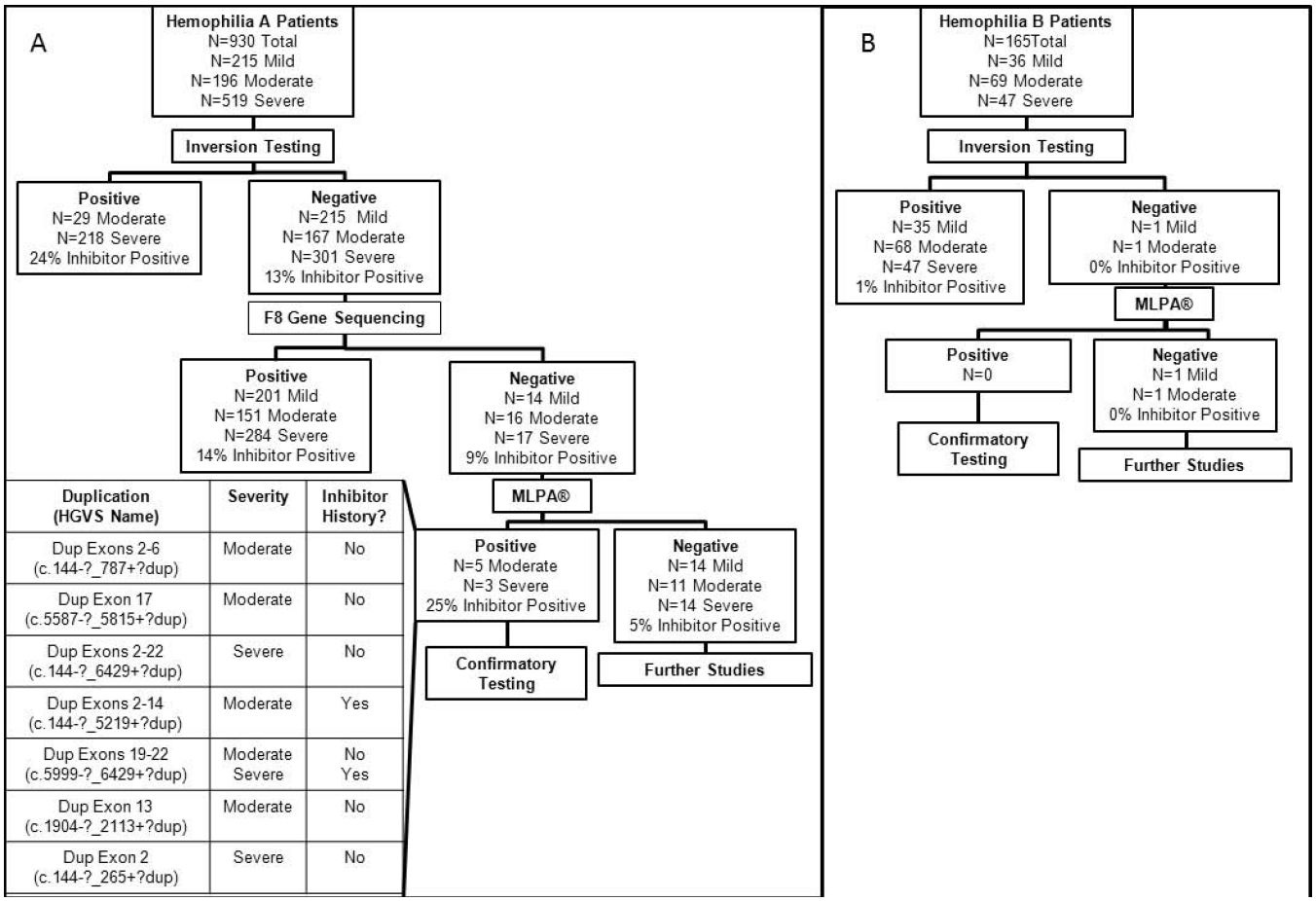

Fig 1.

Mutation screening algorithms incorporating MLPA® a: Mutation screening algorithm for HA patients enrolled in the Hemophilia Inhibitor Research Study b: Mutation screening algorithm for HB patients enrolled in the Hemophilia Inhibitor Research Study