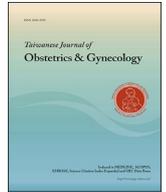




Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

Assessment of 6 STR loci for prenatal diagnosis of Duchenne Muscular Dystrophy



Linh Thuy Dinh ^{a,1}, Van Khanh Tran ^{a,1}, Long Hoang Luong ^a, Phuong Thi Le ^a, Anh Duy Nguyen ^c, Bang Suong Thi Nguyen ^e, Dung Vu Chi ^d, Thinh Huy Tran ^{a,b}, The-Hung Bui ^{a,f}, Thanh Van Ta ^{a,b}, Duc Hinh Nguyen ^{b,*}

^a Centre for Gene and Protein Research, Hanoi Medical University, Hanoi, Viet Nam

^b Hanoi Medical University Hospital, Hanoi, Viet Nam

^c Hanoi Obstetrics & Gynecology Hospital, Hanoi, Viet Nam

^d National Children Hospital, Hanoi, Viet Nam

^e Ho Chi Minh University of Medicine and Pharmacy, Ho Chi Minh City, Viet Nam

^f Center for Molecular Medicine, Clinical Genetics Unit, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

ARTICLE INFO

Article history:

Accepted 4 March 2019

Keywords:

Duchenne muscular dystrophy

Dystrophin

STR analysis

Prenatal diagnosis

ABSTRACT

Objective: Duchenne Muscular Dystrophy is an X-linked recessive disorder characterized by progressive muscular degeneration, patients often develop cardiac failure in the later stage and death occurs before 20 years of age. For a disease with poor postnatal prognosis such as Duchenne Muscular Dystrophy (DMD), providing the carrier mother with the option of prenatal diagnosis in a subsequent pregnancy is accepted practice in many places where termination of pregnancy is allowed. Though methods of direct sequencing such as Sanger's sequencing has been widely used, Next-Generation Sequencing is being increasingly replacing most of its application. For the DMD gene, being the longest gene in the human genome, methods of direct sequencing is often unpractical and time-consuming, instead, STR analysis for linkage analysis would be a cost-effective option and have been used routinely for prenatal diagnosis of DMD. The diagnostic significance of the STRs is based on several criteria, the most important one being the heterozygosity of the locus, power of discrimination (PD) and power of exclusion (PE).

Material and methods: In this study, we investigated the feasibility of application and diagnostic value of 6 STR loci (DSTR49, DSTR50, DXS1036, DXS1067, DXS890, DXS9907) in the proximity of the DMD gene, 66 healthy individuals were recruited for STR analysis and 5 cases of prenatal diagnosis for carrier mother were performed.

Result: Allele frequency, heterozygosity, polymorphic information content, the power of discrimination and exclusion and Hardy–Weinberg equilibrium were analyzed and calculated for the 6 STR loci. 5 of these loci (DSTR49, DSTR50, DXS1067, DXS890, DXS9907) were found practical and useful for preimplantation Genetic diagnosis (PGD) and prenatal diagnosis. All 5 cases of prenatal diagnosis using the method had informative STR results and correct diagnosis.

Conclusion: We concluded that our protocol of STR analysis can be applied for prenatal diagnosis and pre-implantation genetic diagnosis of DMD with high confidence and accuracy, especially in clinical settings where diagnostic resources are more limited.

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Introduction

Duchenne Muscular Dystrophy (DMD, OMIM #310200; ORPHA:98896) is an X-linked recessive disorder characterized by progressive muscular degeneration, patients often develop cardiac failure in the later stage that leads to premature death [1]. Early diagnosis of DMD in the patients does not improve survival or life

* Corresponding author. Center for Gene and Protein Research, Hanoi Medical University, 1st Ton That Tung Street, Hanoi 10000, Viet Nam. Fax: +84 4 38525115.

E-mail addresses: tranvankhanh@hmu.edu.vn (V.K. Tran), duchinh@hmu.edu.vn (D.H. Nguyen).

¹ These authors contributed equally to this work.

expectancy, even with intensive support and healthcare [2]. Therefore, the identification of high-risk individual – carrier females, provision of genetic counseling, and the application of prenatal diagnosis or pre-implantation diagnosis is of great importance in a couple at-risk to allow prenatal diagnosis where it can be offered and thereby reducing the incidence of DMD [3].

For carrier female undergoing pregnancy, prenatal diagnosis is the recommended procedure for confirmation of the disease status of the fetus. The procedure has been widely adopted and accepted by the couple at-risk [4]. Pre-implantation genetic diagnosis (PGD), which requires in vitro fertilization (IVF), genetic analysis and selection of unaffected embryo(s) before the transfer is another option where available. This ensures the transfer of embryo(s) free of the targeted disease. The embryos are biopsied at either day 3 (cleavage stage) or day 5/6 (blastocyst stage) to extract for genetic material for PGD. The low amount of DNA material thus obtained would undergo an extra Whole Genome Amplification (WGA) step to create more DNA templates for analysis [5,6].

Duchenne Muscular Dystrophy is caused by pathogenic variants in the *DMD* gene, located on the X chromosome, spanning 2.4 Mega base-pairs and consists of 79 exons; it is the largest gene in the human genome [7]. Approximately 65% of DMD patients show deletions of one or more exons of the gene, while duplications (about 5%) and point mutations (about 30%) account for the remaining cases [Aartsma-Rus A, Van Deutekom J, Fokkema I et al., 2006. Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. Muscle and Nerve 34, 135–144]. Due to the extreme size, methods of direct sequencing are often unpractical, therefore, STR analysis has been used routinely for prenatal diagnosis of DMD [8], especially for PGD. The diagnostic significance of the STRs is based on several criteria, the most important one being the heterozygosity at the locus [9]. In this study, we investigated the feasibility of application and diagnostic value of 6 STR loci (DSTR49, DSTR50, DXS1036, DXS1067, DXS890, DXS9907) in the proximity of the *DMD* gene and applied these STR markers in 5 cases of DMD prenatal diagnosis.

Method

Subjects and sample collection

We recruited 66 healthy unrelated individuals for STR analysis and assessment and 5 carrier women who were pregnant and opted to have prenatal diagnosis. All women gave informed consent to participate in the study, the study protocol had been approved by the ethical committee of Hanoi Medical University (Hanoi Medical University Institutional Review Board, Hanoi, Vietnam, reference number: IRB00003121).

Samples of 2 ml peripheral blood were obtained from the participants. Fetal samples for prenatal diagnosis were obtained by amniocentesis at 17 weeks of gestation, 10 ml amniotic fluid was used for Karyotyping and 5 ml for STR analysis. DNA was extracted using the ReliaPrep™ Blood gDNA Miniprep System (Promega, Wisconsin, USA).

PCR amplification and electrophoresis

Primers were selected and designed specifically for this study, all primer sequence used are displayed in Fig. 1. Amplification mix was performed using TaKaRa PCR kit (Takara Bio, USA), PCR reaction done on the Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). Cycling conditions were universally [94°–2', (94°–30s, 59°–30s, 72°–30s) x35 cycles, 72°–5'].

Electrophoresis of the amplified product was performed on the GenomeLab GeXP (Beckman Coulter, CA, USA) and analyzed on the system's fragment analysis software.

Prenatal diagnosis of fetuses with a high risk of DMD

Amniocentesis was performed at 17 weeks gestation for women who are heterozygous carrier of *Dystrophin* gene mutation. Sexing of the fetus were done using QF-PCR (Quantitative Fluorescent – Polymerase Chain Reaction). Female fetuses were excluded from further diagnostic workup.

Five fetuses were confirmed by QF-PCR to be male, in which we continued with STR analysis. In conjunction, we also utilized MLPA (Multiplex Ligation-dependent Probe Amplification) (MRC Holland, Amsterdam, Netherlands) to verify the diagnostic accuracy of the STR analysis.

Statistical analysis

Data entry were done according to the STRAF-A format and analyzed using “STRAF 1.0.5: STR Analysis for Forensics” tool [10]. The dataset used for analysis can be provided with the reasonable request to the authors.

Result

The heterozygosity of the six STRs was calculated and presented in Table 1. We categorize heterozygosity by the expected and observed value. Two loci with the highest heterozygosity were DSTR49 (Expected: 0.866, Observed: 0.864) and DXS890 (Expected: 0.7461, Observed: 0.7424). The allele frequencies of the 6 STRs were represented in Fig. 1. The number of alleles differed between individual STR locus, with DSTR49 having 14 alleles, other loci ranging between 4 and 9 alleles. Allele frequencies of the loci varied from 0.01 to 0.49.

We also calculated other statistical value of the six STRs including PIC: Polymorphic Information Content, PD: Power of discrimination, PE: Power of elimination, GD: Genetic diversity and PM: Probability of random match to assess the diagnostic power of these loci (Table 2).

The expected heterozygosity of at least 2 STRs when using 5 STRs (DSTR49, DSTR50, DXS1067, DXS890, DXS9907) was 97.76%, which meet the statistical requirement for an application in Prenatal Diagnosis and Pre-implantation genetic diagnosis. Using this method, we have performed prenatal diagnosis on 5 cases where the woman was DMD pathogenic allele carrier. In all the 5 cases we were able to discern the pathogenic allele with at least 2/5 informative STRs. An example case of prenatal diagnosis is presented in Fig. 2.

Discussion

Prenatal diagnosis and more recently pre-implantation genetic diagnosis have been core elements in the management of genetic disorder in pregnancy. However, one of the main errors in prenatal diagnosis is due to maternal blood contamination during amniocentesis; this leads to a false positive by co-amplification of the maternal gene. This problem can be addressed by the use of STR for linkage analysis. For DMD, due to the disease characteristic and the size of the *DMD* gene, STR analysis had been a preferred approach of assessing disease status of the fetus. Traditionally, the process of selecting STR loci for analysis was mainly based on experience and referral from previous publications. However, due to the difference in population genetics, using STRs from the study of other populations is not always feasible and can yield insufficient

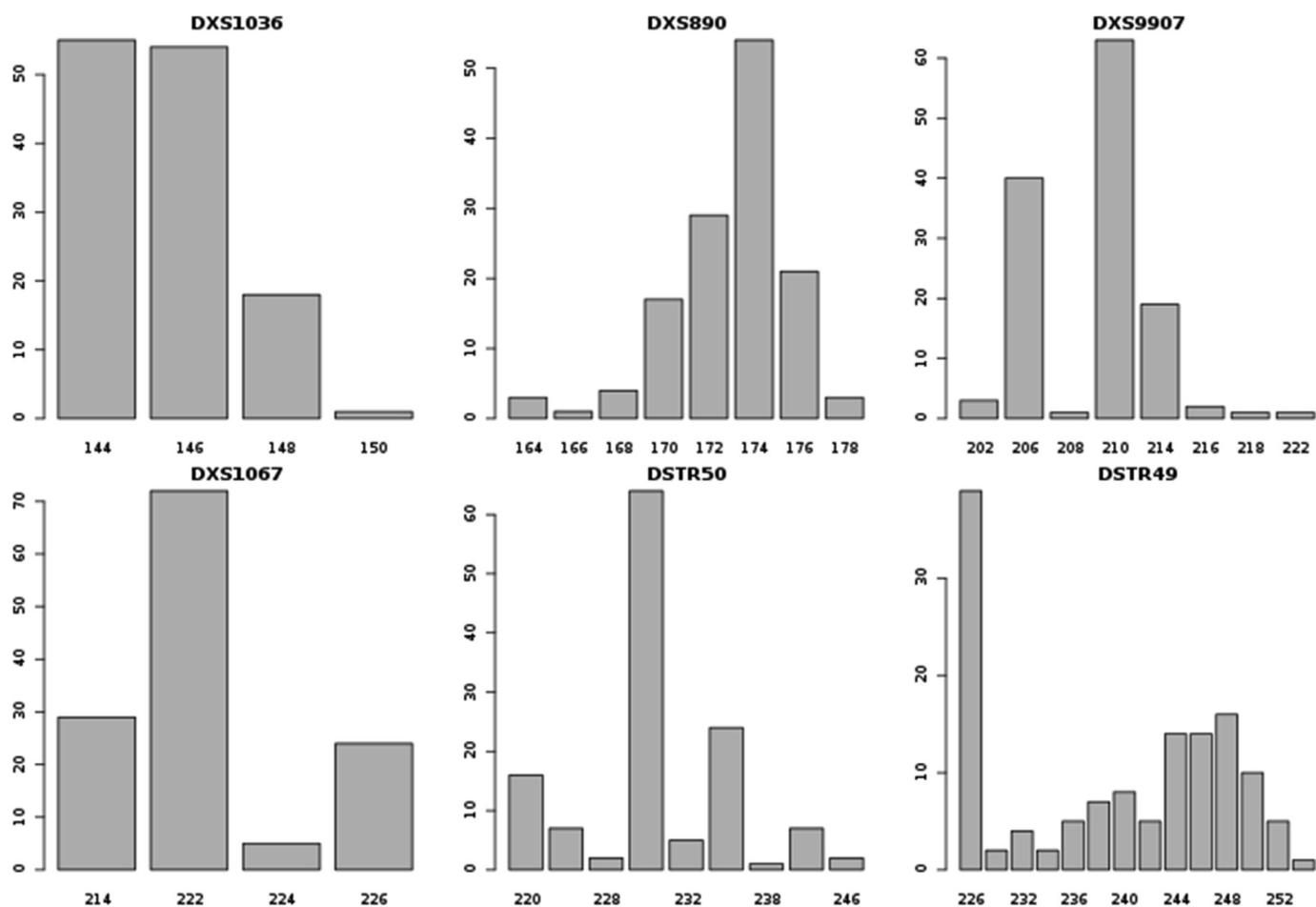


Fig. 1. Allele frequency of the six STR loci.

Table 1

Primers used for STR analysis in the study.

STRs	Dye	Location	Forward Primer	Reverse primer
DXS8090	Green	Intron 1	GGGTGAAATTCATCAAAA	ACAAATGCAGATGTACAAAAATA
DXS9907	Green	Intron 45	CTGTGGTGTAAAGGTTGCTT	TAGACTTGACCTCATGGCT
STR49	Blue	Intron 49	CGTTACCAGCTCAAATCTCAAC	CATATGATACGATTCGTGTTTGC
DXS1067	Green	Intron 50	TATGTCTCAGACTATTCAGATGCC	CTCCAGTAACAGATTTGGGTG
STR50	Blue	Intron 50	AAGGTTCTCCAGTAACAGATTTGG	TATGCTACATAGTATGCTCAGAC
DXS1036	Blue	Intron 51	TGCAGTTTATTATGTTCCACG	GCCATTGATAAGTGCCAGAT

Table 2

Heterozygosity index and an exact test for Hardy–Weinberg equilibrium of the STRs.

Locus	N	No. Allele	Expected Heterozygosity (Genetic diversity)	Observed Heterozygosity	pHW
DSTR49	132	14	0.866	0.864	0.393
DSTR50	128	9	0.697	0.625	0.005
DXS1036	130	4	0.634	0.431	<0.001
DXS1067	130	4	0.613	0.615	0.762
DXS890	132	8	0.746	0.742	0.130
DXS9907	130	8	0.653	0.631	0.146

information for prenatal diagnosis. Furthermore, even with recent technology such as Next-generation sequencing in PGD which allow for direct confirmation of the disease status of the embryo, STR analysis is still needed to be used in conjunction to address the problem of Allele dropout (ADO). ADO could be seen in 5–20% of blastomeres sample amplification and a relatively common cause of

misdiagnosis. STR analysis would help in the interpretation of the result and lead to a higher success rate for the PGD procedure (see Table 3).

In this study, we have demonstrated the process of selection and verification of six STRs, based on the calculated heterozygosity and statistical diagnostic values as calculated by the bioinformatics tool.

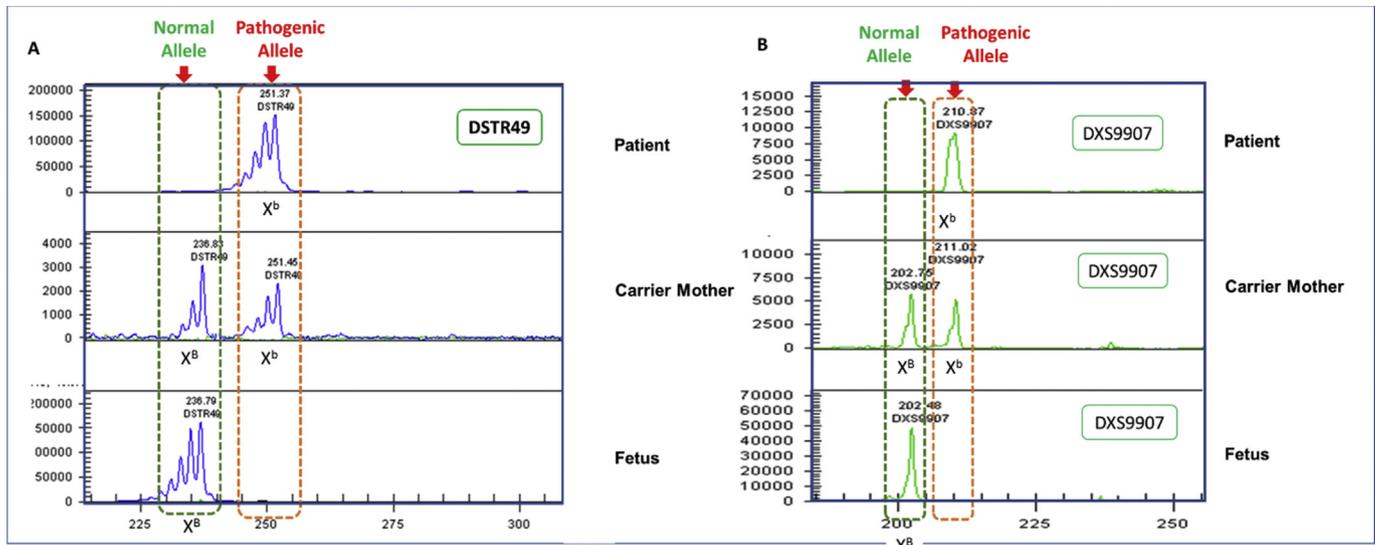


Fig. 2. Prenatal diagnosis case with DSTR49 and DSTR50 being heterozygous and indicative for the disease-causing allele.

Table 3
Diagnostic value of the STRs.

Locus	No.	No. allele	PIC	PM	PD	PE
DSTR49	132	14	0.848	0.044	0.956	0.722
DSTR50	128	9	0.660	0.149	0.851	0.322
DXS1036	130	4	0.553	0.212	0.788	0.134
DXS1067	130	4	0.553	0.206	0.794	0.310
DXS890	132	8	0.704	0.117	0.883	0.497
DXS9907	130	8	0.589	0.1934	0.8066	0.329

PIC: Polymorphic Information Content.

PD: Power of discrimination.

PE: Power of elimination.

PM: Probability of random match.

Based on heterozygosity, the locus DSTR49 had the highest indicative value (Expected: 0.866, Observed: 0.864, p -HW: 0.393), followed by DXS890. Four STRs meet the Hardy–Weinberg equilibrium (DSTR49, DXS1067, DXS890, DXS9907); DSTR50 and that DXS1036 did not meet HWE was probably due to low genetic diversity in these two loci (low number of the allele, low observed heterozygosity) or the occurrence of genetic drifts. Similarly, other statistical values were evaluated; DSTR49 had the highest PIC, PD and PE (0.848, 0.956, and 0.722 respectively) and the lowest Probability of random match (PD: 0.044). On the contrary, for DXS1036, due to the low PD (0.788) and PE (0.134), we concluded that it has low discriminative value thus we only included 5 STRs for the prenatal diagnosis protocol (DSTR49, DSTR50, DXS1067, DXS890, DXS9907).

The standard criteria for prenatal diagnosis would require at least 2 STR markers to be heterozygous. Based on the expected heterozygosity, we calculated the likelihood of at least 2/5 STR to be heterozygous to be 97.76%. This value is sufficient for standard prenatal diagnosis protocol. For verification of the prenatal diagnosis protocol, all our 5 cases which underwent prenatal diagnosis had at least 2/5 heterozygous STRs which were sufficient to ensure correct prenatal diagnosis. The amniocenteses were performed at week 17 gestation, amniotic fluid was taken for STR analysis and with the protocol, the result was returned after 3 days. The quick return of diagnostic information in these cases would allow easier decision making for the mother and obstetrician. There was no conflict between the STR analysis result and the MLPA result. We

would suggest the prenatal diagnosis protocol as follow: (1) Sexing of the fetus and continue the diagnostic work-up for male fetuses; (2) Utilize method to detection of *Dystrophin* mutation: STR analysis and/or MLPA. It is suggested that for microdeletion or microduplication mutation it would be simpler and diagnostically safer to prioritize MLPA over STR analysis for prenatal diagnosis. However, using both methods in prenatal diagnosis might prove advantageous as it increases the validity of the results.

In conclusion, the protocol was suitable and efficient in prenatal diagnosis and furthermore would be applicable for PGD of DMD. The 5 markers selected for the protocol had high diagnostic confidence and accuracy.

Conflict of interest

The authors declare no conflict of interest.

Author's contribution

TVT, DHN, LTD, VKT coordinated the study. LTD, VKT, LHL designed the study. LTD, BSTN, DVC provided patient care and collected data; LHL, PTL, AND, THT performed genetic analysis. LHL, THT, THB performed statistical analysis. LTD, LHL, THB interpreted the results and wrote the manuscript. All authors critically reviewed the report. No writing assistance was provided. The corresponding author had full access to all of the data in the study and take responsibility for the integrity of the data. All authors revised the manuscript critically and approved the final version for publication.

Availability of data and materials

The dataset and material of this study are available from the corresponding author on reasonable request. No identifiable information of the patients would be disclosed.

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