Identification of deletions and duplications in Ecuadorian patients with Duchenne Muscular Dystrophy

Paola Cristina Montenegro Larrea

The University of Nottingham
School of Molecular Medical Sciences
Human Genetics Research Laboratory
Queen’s Medical Centre Campus
A Floor, West Block

Supervisor: Dr Sally Chappell

This dissertation is submitted in partial fulfilment of the project requirements for the MSc in Molecular Genetics and Diagnostics

August, 2013

Summary: 247 words Number of tables: 1
Main text: 4425 words Number of figures: 9

STUDENT Declaration 1: I declare that all the work presented in this dissertation is my own, except where otherwise stated.

Date: ……………………… Student signature: …………………………………………..

SUPERVISOR Declaration 2: I have seen this submission and the work described is the student’s own or is acknowledged accordingly

Date: ………………………Supervisors signature: …………………………………………..
SUMMARY

Duchenne muscular dystrophy is an X linked recessive genetic disorder caused by mutations in the dystrophin gene (DMD). According to the Public Health Ministry of Ecuador, DMD affects 20 to 30 in every 100,000 born males. During the last decade, there has been an important investment in the development of biomedical sciences in Ecuador, which leads to research and implementation of new molecular diagnostic technologies that will help to improve the Ecuadorian health system. This is the first research project that proposes multiplex ligation-dependent probe amplification or MLPA as a complementary molecular diagnostic technique for the screening of deletions and duplications in the DMD gene in Ecuadorian patients clinically diagnosed with DMD. From the 16 patients analysed with MLPA, three male patients showed a deletion cluster of exons 45 to 50 in the central region of the gene, commonly related with the disease. Interestingly, a duplication of exons 53 to 57 was found in one of the patients with no positive DMD carrier pattern in the mother, a de novo mutation reported as an inherited DNA disorder in the DMD databases. Even though no female carrier status was found, the technique allows not only to identify new deletions or duplications, but also is able to determine the maternal origin of the disease. It is conclude that MLPA is an adequate technique with accurate, rapid and high quality results that can help to determine the main deletions and duplications that cause Duchenne muscular dystrophy in the Ecuadorian population.

INTRODUCTION

Duchenne muscular dystrophy (OMIM 310200) is recognized as the most severe form of the muscular dystrophies related with mutations in the dystrophin gene (DMD) [1]. The life expectancy of affected individuals averages between 19 to 25 years, commonly with cardiorespiratory complications at the end. DMD genetic impairment is an X linked recessively inherited disease, muscle debilitating and degenerative, with an estimated incidence of 1 in 3500 to 6000 live male births [2]. In humans, the DMD gene is considered as the largest gene in the genome. With 2.4Mb, the DMD gene is located in the short arm of the X chromosome in the Xp21 locus, and contains 79 exons. In normal conditions the DMD gene codes for dystrophin, a 400kDa membrane protein involved in the stability of the membrane structure, mainly in the skeletal muscle cells [3]. Absence of dystrophin protein, which connects the cytoskeleton to the external basement membrane, promotes myocyte necrosis and subsequent fibrosis, which may lead from progressive muscle weakness to left ventricular dilation and failure [4]. Clinically, Duchenne muscular dystrophy affects mostly boys that are apparently normal at birth. Progressively the patients show a delayed
independent ambulation, tiptoe walking and delayed speech. In general, a proximal muscle weakness precedes a distal weakness. Most of the patients die in their early 20s from cardio-respiratory failure [3].

Diagnosis of Duchenne muscular dystrophy is a complementary procedure that involves both clinical and molecular analysis. The lack of dystrophin in the cell membrane leads to the release of creatine kinase (CK) from the cytoplasm of myocytes, allowing the diagnosis to be made by markedly increased serum CK levels [3]. Clinical laboratory tests determine that normal CK values are usually between 60 and 400 units/L, where one unit represents the enzyme activity necessary to catalyse 1 μmol of substrate per minute under specific conditions. Before symptoms and signs develop, serum CK levels measurements can exceed the 400 units/L normal threshold and be over 1000 units/L and beyond [5]. This procedure does not define the disease as Duchenne since the milder Becker muscular dystrophy is also related with high CK levels in blood [6]. On the other hand, the molecular diagnostic test is based on screening of the dystrophin gene for deletions, duplications or point mutations, which will confirm the presence of an abnormal or absent dystrophin protein. The origin of DMD mutations depends on the type of the change. For instance, it is most likely that point mutations originally come from errors in spermatogenesis; meanwhile, deletions and duplications rise from a defective oogenesis [7]. The resulting aberrant dystrophin protein arises in around 70% of the cases from frameshift deletions and duplications, but also from missense and nonsense point mutations in a lower rate, approximately in 30% of the cases [8,9,10].

The molecular diagnostic techniques are useful to determine the disease as DMD. The classic DMD molecular diagnostic is a relatively simple multiplex PCR that amplifies only 18 from the 79 exons in the gene, reported as deletion hot spots in the central region of the gene [11]. However, as this method is based on detecting the absence of a PCR product to show a deletion, it fails to detect duplications, intronic changes and point mutations, and it is unable to characterise deletion breakpoints. In addition, the DMD multiplex PCR is limited by the size of the gene and cannot be used for carrier testing of females [4].

Currently, the DMD 18 exon hot spot multiplex PCR is a technique used frequently in molecular diagnostics laboratories as a first screening of the DMD gene. The multiplex PCR will detect DMD in 65% of the reported cases [4], which means that ~35% of DMD molecular causes can be related to duplications, point mutations or even deletions of any of the 61 exons that are not been screened by the classical technique.
In Ecuador, according to the Public Health Ministry, DMD affects 20 to 30 in every 100,000 born males. In 1999, Paz y Miño and colleagues reported the first multiplex PCR analysis of the Dystrophin gene in five Ecuadorian patients [12], and they found a common deletion between exons 45 and 51 in three symptomatic patients. Since then, no further research has been done in Ecuadorian patients with DMD. Some molecular diagnostics laboratories have adopted the classical PCR to offer a partial diagnostic of DMD. For instance, the Molecular Genetics Laboratory at the Hospital de Especialidades de las Fuerzas Armadas del Ecuador (Ecuadorian Army Hospital), one of the main health care centres in the country, standardized the technique and has offered the diagnostic test since 2009. Around 30 patients have been screened for big deletions in the 18 exon hot spots of the DMD gene, and only one patient presented a deletion of exons 45 to 52. This raises the hypothesis that maybe the Ecuadorian patients do not share the same deletion hotspots reported in the literature, and due to their mixed ancestry, the related symptoms with Duchenne muscular dystrophy in Ecuadorian patients may be caused by deletions in different exons, or other different mutations.

The aim of this research project is to apply a technique that allows all 79 exons of the DMD gene to be screened for deletions and duplications in Ecuadorian DNA samples previously analysed with the DMD multiplex PCR. The Multiplex Ligation-dependent Probe Amplification or MLPA, is an accurate technique that offers a rapid deletions and duplications diagnostic of the dystrophin gene. It is the most widely used technique to detect exonic deletions and duplications mutations, as a sensitive and discriminative tool, MLPA can be used for the analysis of all 79 DMD exons [4].

Basically, the MLPA technique combines the power of PCR with the specificity of probe hybridization and capillary electrophoresis. The MLPA methodology, developed by Schouten and colleagues in 2002, is able to quantify up to 40 different exonic DNA sequences in a very straightforward reaction to establish a copy number variation of a specific gene. The reaction requires mixing high quality DNA (~20ng/µl) with specific probes that consist of two oligonucleotides, one synthetic and one M13 derived. The amplification will depend on the hybridization and ligation of the two-oligonucleotide probe to specific sites of the target sequence. The different probes have identical end sequences, allowing a PCR amplification with only one pair of primers (Figure 1) [13].
The PCR products, between 130 and 480bp, are separated using high resolution capillary electrophoresis. The resulting electropherogram will provide the size of the fragments and fluorescent peak heights that represent each exonic region. In accordance with the National Genetics Reference Laboratory (NGRL) of Manchester, peak heights appear to be a simpler and therefore more consistent measure than peak area. Comparisons between the peak heights and peak areas as measures of peak intensity have shown that the variance of peak area measurements is consistently higher than those for peak heights [14]. Spreadsheets offered by the NGRL are useful to normalise data using a linear regression model based on the degree of sloping of the control ligation products. These worksheets analyse data to produce dosage quotients (DQs) against normal standards [14]. Any difference of DQ will determine the absence or duplication of each exon.

The employment of MLPA in the search of deletions and duplications in the whole DMD gene in the Ecuadorian DNA samples not only will prove the effectiveness of the technique in the Duchenne muscular dystrophy diagnosis, but also will reveal the possible presence of different mutations that may affect Ecuadorian patients and other Latin-Americans. Determining the pattern of genetic diseases that rule over the Latin-American people can allow to create more accurate diagnostic strategies and treatments specifically addressed to their mixed ancestry.
MATERIALS AND METHODS

Patients

All 16 patients came from different regions of Ecuador (Table 1). There were 11 male patients ranging between 3-16 years old, and 5 female patients as possible carriers between 14-40 years old. Male patients were clinically diagnosed with Duchenne muscular dystrophy during June 2009 to August 2012 by clinical geneticist Rosario Paredes M.D. from Hospital General de Especialidades de las Fuerzas Armadas (Ecuadorean Army Hospital). The DMD diagnostic criteria included the following clinical details: 1. Physical Examination - age of onset, gait instability acquired through the years, calf muscle hypertrophy and Gowers’ sign positive as an indicative of proximal muscle weakness (This sign describes a patient that has to use his hands and arms to walk up his own body from a squatting position due to lack of hip and thigh muscle strength [15]). 2. Pedigree - X linked inheritance pattern of the disease in the family. 3. Laboratory test - Creatine kinase or CK levels in blood extremely elevated (over 400 units/L). 14 of the 16 DNA samples were tested initially, by the Molecular Genetics Laboratory in Quito, using multiplex PCR for the screening of the promoter region (Pm) and exons 3,4,6,8,12,13,17,19,43,44,45,47,48,50,51,52,60. Only patient 9 showed a deletion cluster of exons 45 to 52 by multiplex PCR; this sample was also included for confirmation using MLPA analysis.

Table 1. Patients Data

<table>
<thead>
<tr>
<th>DMD code</th>
<th>Test Date</th>
<th>Clinical Diagnostic</th>
<th>Gender</th>
<th>Age</th>
<th>Place of Birth</th>
<th>PCR Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20/06/2009</td>
<td>DMD</td>
<td>M</td>
<td>4</td>
<td>Quito</td>
<td>Non tested</td>
</tr>
<tr>
<td>2</td>
<td>20/06/2009</td>
<td>DMD</td>
<td>M</td>
<td>7</td>
<td>Quito</td>
<td>Non tested</td>
</tr>
<tr>
<td>3</td>
<td>25/02/2010</td>
<td>DMD Carrier</td>
<td>F</td>
<td>33</td>
<td>Quito</td>
<td>No deletions</td>
</tr>
<tr>
<td>4</td>
<td>02/09/2010</td>
<td>DMD</td>
<td>M</td>
<td>16</td>
<td>Quito</td>
<td>No deletions</td>
</tr>
<tr>
<td>5</td>
<td>12/11/2010</td>
<td>DMD Carrier</td>
<td>F</td>
<td>14</td>
<td>Quito</td>
<td>No deletions</td>
</tr>
<tr>
<td>6</td>
<td>30/11/2010</td>
<td>DMD</td>
<td>M</td>
<td>14</td>
<td>Quito</td>
<td>No deletions</td>
</tr>
<tr>
<td>7</td>
<td>11/01/2011</td>
<td>DMD</td>
<td>M</td>
<td>4</td>
<td>Quito</td>
<td>No deletions</td>
</tr>
<tr>
<td>8</td>
<td>09/06/2011</td>
<td>DMD Carrier</td>
<td>F</td>
<td>26</td>
<td>Quito</td>
<td>No deletions</td>
</tr>
<tr>
<td>9</td>
<td>24/08/2011</td>
<td>DMD</td>
<td>M</td>
<td>3</td>
<td>Quito</td>
<td>Del Exons 45, 47, 48, 50, 51, 52</td>
</tr>
<tr>
<td>10</td>
<td>10/05/2012</td>
<td>DMD Carrier</td>
<td>F</td>
<td>40</td>
<td>Quito</td>
<td>No deletions</td>
</tr>
<tr>
<td>11</td>
<td>10/05/2012</td>
<td>DMD</td>
<td>M</td>
<td>6</td>
<td>Daule</td>
<td>No deletions</td>
</tr>
<tr>
<td>12</td>
<td>15/06/2012</td>
<td>DMD</td>
<td>M</td>
<td>7</td>
<td>Guayaquil</td>
<td>No deletions</td>
</tr>
<tr>
<td>13</td>
<td>06/07/2012</td>
<td>DMD</td>
<td>M</td>
<td>14</td>
<td>Puerto Bolivar</td>
<td>No deletions</td>
</tr>
<tr>
<td>14</td>
<td>27/07/2012</td>
<td>DMD</td>
<td>M</td>
<td>7</td>
<td>Latacunga</td>
<td>No deletions</td>
</tr>
<tr>
<td>15</td>
<td>07/08/2012</td>
<td>DMD</td>
<td>M</td>
<td>3</td>
<td>Guayaquil</td>
<td>No deletions</td>
</tr>
<tr>
<td>16</td>
<td>22/08/2012</td>
<td>DMD Carrier</td>
<td>F</td>
<td>ND</td>
<td>Manta</td>
<td>No deletions</td>
</tr>
</tbody>
</table>

ND: NO DATA

DNA extraction

Blood samples with EDTA (3ml) were collected from patients in Ecuador and DNA was extracted using the High Pure PCR template preparation kit (Roche), following the handbook protocol. Purified DNA was stored at -20°C until tested. Informed consent was
obtained from each patient/parent towards genetic diagnostic and utilization of the samples and results for research purpose. DNA samples were sent from Quito-Ecuador to Nottingham-UK via FEDEX special currier in cold clinical pack. DNA stock concentrations ranged from 23 to 213ng/µl. Where possible, 20µl aliquots were diluted in 1x TE to a final working concentration of 50ng/µl required for the following MLPA reaction.

**Multiplex Ligation-dependent Probe Amplification (MLPA)**

MLPA analysis was carried out using Salsa probemix PO34 (exons 1 to 10; 21 to 30; 41 to 50 and 61 to 70) and Salsa probemix PO35 (Dp427c, 11 to 20, 31 to 40, 51 to 60 and 71 to 79), in two separate sessions respectively. In addition, required MLPA reagents were purchased commercially from MRC Holland, Amsterdam. The standard operating procedure (SOP) and training were provided by the Molecular Genetics Service from Nottingham University Hospitals NHS Trust. All thermal reactions were performed on an Applied Biosystems Verity® 96-well standard thermal cycler. As far as possible, 5µl of 50ng/µl of high concentrated DNA samples were denatured at 98°C for 5min. For those samples with concentrations were lower than 50ng/µl, 5µl of DNA were taken directly from the stock. Immediately, the samples were incubated overnight at 60°C (approx. 16h to 20h) with 3µl MLPA probe mix (1.5µl probemix (P034 and P035 respectively) plus 1.5µl of new MLPA buffer in a 1:1 proportion), this will ensure the specific hybridisation of the oligonucleotide probes with their target sequences. Samples were then treated with 32ul Ligase 65 reaction mix (1µl Ligase 65, 3ul Ligase 65 buffer A, 3ul Ligase 65 buffer B and 25ul dH2O) for 15min at 54°C. The ligase reactions were stopped by incubation at 98°C for 5 min. Finally, fluorescent PCR amplification was carried out with the specific 2µl SALSA FAM PCR primers (10pmol), 0.5µl Salsa Polymerase and 7.5µl dH2O under the following thermocycling conditions: 33 cycles of 95°C for 30sec, 60°C for 30sec, 72°C for 1min, and a final extension of 72°C for 20min.

**Capillary Electrophoresis**

Amplification products were analysed by capillary electrophoresis on an ABI PRISM 3130 Genetic Analyser (Applied Biosystems, USA) with the following modules: capillaries 36 cm, Polymer POP-7, run temperature 60°C, pre-run voltage 15 kV, pre-run time 180 sec, injection voltage 1.2 kV, injection time 16 sec, run voltage 15 kV, data delay time 60 sec, run time 1200 sec. The obtained peaks were analysed by using Genemapper 3.7 Software. Five healthy males were analysed as normal controls, one affected female with a deletion of exons 10-21 was used as a unique positive control for both probemixes, and one water sample was included as a control to detect possible DNA contamination. 1.5µl of each PCR
product was diluted with 12µl of Hi-Di formamide (approx. 1:12), in addition with 6µl of GS500-LIZ internal size standard before being subjected to electrophoresis.

**Analysis of MLPA data**

The Genemapper results were exported to an excel sheet in a data table that was organized based on exon IDs and peak heights for each probemix. The peak heights were used to calculate the dosage quotient (DQs) using the National Genetics Reference Laboratory MLPA Spreadsheet PO34 and PO35 (NGRL, Manchester-UK). The NGRL Manchester spreadsheets were Excel macros that compared peak heights with a positive internal control and with peaks of 5 normal control samples. This software determined the relative probe signals of each probe by dividing each measured peak height (Hs) by the sum of all 45 peaks heights (ΣHs) of that sample. The relative peak area (Hs/ΣHs) was then divided by the relative peak height of the corresponding probe obtained from a control DNA sample (Hc/ΣHc). Results are given in terms of normalized DQs and normalized peak heights [16]. The normal DQ ranges from 0.8 to 1.2, for all those values under or above the normal DQ range will confirm the presence of deleted or duplicated fragments respectively.

The P034 DMD probemix contained 45 different probes with amplification products between 129 and 490bp, as well as 10 control fragments generating an amplification product smaller than 120bp. The control fragments included two (105-118bp) chromosome Y-specific probes. Likewise, the P035 DMD probemix contained 45 different probes with amplification products between 129 and 490bp, as well as 9 control fragments generating an amplification product smaller than 120bp. The control fragments included one (105bp) chromosome Y-specific probe [17]. Each probe generated a specific peak patterns during the capillary electrophoresis, which are used as a reference of a good PCR performance and as a first hint of the presence of possible deletions and duplications (Figs. 2 and 3).
Figure 2. Capillary Electrophoresis normal male pattern for SALSA MLPA probemix P034. The red arrow shows the specific Y male probe, absent in women samples. (Modified from [17])

Figure 3. Capillary Electrophoresis normal male pattern for SALSA MLPA probemix P035. The red arrow shows the specific Y male probe, absent in women samples. (Modified from [17])
RESULTS

**Determination of DMD patients**

Clinically, all male patients showed difficulties to walk, to stand up and recurrent falls at an early age. Physical examination confirmed gait instability acquired through the years, calf muscle hypertrophy and a positive Gowers’ sign. A pedigree analysis of the 10 young male patients physically diagnosed with Duchenne muscular dystrophy revealed that 5 of them had a positive DMD family history. The patients that had access to a CK test presented values over 1000 units/L, which indicated the presence of a myopathy. The 13 patients previously analysed by multiplex PCR analysis (Table 1) resulted only in one patient (patient 9) with a deletion cluster from exon 45 to exon 52. No mutations were identified for the other patients. Meanwhile, the PCR results for the 5 possible female carriers were negative for deletions in the Dystrophin gene. The siblings, patients 1 and 2, were not previously tested by multiplex PCR.

**MLPA validation**

The multiplex ligation-dependent probe amplification or MLPA technique was first standardized after one training session at the Molecular Genetics Service from Nottingham University Hospitals NHS Trust. The Molecular Genetics laboratory also provided the normal and the positive controls for MLPA DMD analysis. Six normal, one blank and one positive control samples were used to validate the reproducibility of the protocol. The controls were coded as follows: positive control C+, blank control B, normal control N1, normal control N2, normal control N3, normal control N4, normal control N5 and normal control N6. The MLPA results of the controls showed that all the samples were suitable as controls for the MLPA DMD diagnostic, except for N5. Figures 4 and 5 show that dosage quotients bars of normal controls N1, N2, N3, N4 and N6 are similar in the normal range between 0.8 to 1.2; however, sample N5 presented uneven DQs bars, which may represent a degradation of the DNA. The positive control C+ deletions of exons 10-21 were confirmed with the two probemix assays, which dosage quotient bars were recorded under 0.8 and over 0.4, a 35-50% reduction in relative peak height. This represented a heterozygous carrier female.
Figure 4. MLPA Probemix PO34 dosage quotients (DQs) results: Normal controls (N1, N2, N3, N4, N5, N6) and Positive control (C+ - exons 10 and 21 deleted). Blue DQs bars are chromosomal internal controls of DNA denaturation. Green DQs bars represent DMD exons fragments, all of them between the normal range of 0.8 to 1.2, except for normal control N5 which is not suitable for the DMD MLPA test. Black arrow shows the C+ deletion of exons 10 and 21, a heterozygous female represented by bars under 0.8 and over 0.4.
Figure 5. MLPA Probemix PO35 dosage quotient (DQs) results: Normal controls (N1, N2, N3, N4, N5, N6) and Positive control (C+ - exons 11 to 20 deleted). Blue DQs bars are chromosomal internal controls of DNA denaturation. Normal controls DQs bars in green represent DMD exons fragments, all of them between the normal range of 0.8 – 1.2, except for normal control N5 which is not suitable for the DMD MLPA test. Black arrow shows the C+ deletion of exons 11 and 20, a heterozygous female represented by bars under 0.8 and over 0.4.
**DMD MLPA results in patients**

Patients 1 to 16 were all analysed by MLPA, in two different sessions: one for the Salsa Probemix PO34 (exons 1 to 10; 21 to 30; 41 to 50 and 61 to 70) and another for the Salsa Probemix PO35 (Dp427c, 11 to 20, 31 to 40, 51 to 60 and 71 to 79), a total of 79 exons of the DMD gene. After doing the peak heights dosage quotient analysis, the results showed no deletions or duplications in patients 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14 and 16 which presented dosage quotients in the normal range 0.8 to 1.2 (Figs. 6a,b and 7a,b).

**Figure 6a. MLPA Probemix PO34 dosage quotient (DQs) results:** patients 3, 4, 5, 6, 7 and 8 with no mutation identified. Exons DQs bars in green between the normal range 0.8 to 1.2. Chromosomal control DQs bars in blue indicate a proper DNA denaturation.
Figure 6b. MLPA Probemix PO34 dosage quotient (DQs) results: patients 10, 11, 12, 13, 14 and 16 with no mutation identified. Exons DQs bars in green between the normal range 0.8 – 1.2. Chromosomal control DQs bars in blue indicate a proper DNA denaturation.
Figure 7a. MLPA Probemix PO35 dosage quotient (DQs) results: patients 3, 4, 5, 6, 7 and 8 with no mutation identified. Exons DQs bars in green between the normal range 0.8 to 1.2. Chromosomal control DQs bars in blue indicate a proper DNA denaturation.
Figure 7b. MLPA Probemix PO35 dosage quotient (DQs) results: patients 10, 11, 12, 13, 14 and 16 with no mutation identified. Exons DQs bars in green between the normal range 0.8 to 1.2. Chromosomal control DQs bars in blue indicate a proper DNA denaturation.
Patients 1, 2 and 9 presented two common in-frame deletion clusters of exons located in the central region of the gene (Fig. 8). The deletions were represented by the absence of the dosage quotients bars. Samples 1 and 2 showed a deletion cluster of exons 45 to 50. Patient 9 presented a common deletion fragment of the exons 45 to 52, which confirmed its previous multiplex PCR results. These outcome indicated that the boundaries of these deletions are located at 5’ end between exons 44 and 45 (possible breakpoint on intron 44) and at 3’ ends between exons 50-51, and 51-52 (intuitive breakpoints on introns 50 and 51).

![Figure 8. MLPA Probemix P034 and P035 dosage quotient (DQs) results](image)

**Figure 8. MLPA Probemix P034 and P035 dosage quotient (DQs) results:** patients 1 and 2 with deletion clusters of exons 45 to 50 and patient 9 with deletion clusters of exons 45 to 52. Deletions represented by the absence of DQs green bars. Chromosomal control DQs bars in blue indicate a proper DNA denaturation.

Additionally, the MLPA results showed a duplication in patient 15 that corresponds to the cluster of exons 53-57. In this case, the duplication is a possible *de novo* mutation, since the mother of the patient, sample 16, was negative for deletions and duplications after the MLPA analysis (Fig. 9).
From the 16 Ecuadorian patients analysed by MLPA, 3 of them presented common deletion clusters in the central portion of the DMD gene, this represented the 20% of the cases. One important finding was the apparent de novo duplication of exons 53 to 57, which mutation is not inherited since the mother of the patient did not present this duplication cluster in the DMD gene.

DISCUSSION

This is the first research project that reports the application of the MLPA technique to screen deletions and duplications in 16 Ecuadorian patients clinically diagnosed with Duchenne muscular dystrophy. The methodology was fast and easily standardized, showing from the beginning that the technique is very straightforward for diagnostic purposes. The first results obtained from the controls analysis were ready to be diagnosed in three days. The accuracy of the results and the short time protocol indicate that the proposal of MLPA as a diagnostic strategy is a convenient, fast, precise and cost-effective alternative to the DMD multiplex PCR.

The powerful application of PCR amplification combined with the sensitivity of hybridization and capillary electrophoresis make of the multiplex ligation-dependent probe amplification MLPA one of the most accurate techniques to screen deletions and duplications in human genes. As a result, MLPA is able to detect alterations in gene dosage which is of
great importance for therapeutic, prognostic, or diagnostic purposes [18]. Nowadays, MLPA is widely employed to screen for deletions and duplications in the gene responsible for Duchenne muscular dystrophy (DMD) [4,19,20].

Since the standardization of multiplex PCR protocol to amplify a total of 18 exons [21,22], the PCR technique has evolved in order to overcome limitations related with the number and size of amplification fragments. The molecular diagnostic of DMD by multiplex PCR is able to detect deletion hot spots distributed in the central region of the gene, and can be modified to detect up to 30 exons which represent 98% of the common deletions that alter the gene [20,22,23]. However, the multiplex PCR cannot assess the detection of duplications, or of deletions in female carriers, the former being a cause of Duchenne’s in ~11% of the cases [24]. The evolution of multiplex PCR in the novel MLPA technique presents a high sensitive diagnostic tool for detection of deletions and duplications in the DMD gene with accurate, rapid and high quality results.

In Ecuador, DMD multiplex PCR has been applied as a molecular diagnostic strategy since the late 1990s. However, the screening of deletions in the DMD gene by multiplex PCR covers only ~65% of all the mutations that can cause Duchenne muscular dystrophy [22]. Ecuadorian medical genetics centres have had a relatively late development compared with other Latin American countries [25], thus the development and introduction of new molecular techniques represented a large investment of time and money. Recently, health authorities are showing a considerable interest in medical sciences and the Ecuadorian government is willing to support the development of human molecular genetics services. The implementation of MLPA as a novel molecular diagnostic strategy to detect deletions and duplications in the DMD gene of the Ecuadorian population is a reliable possibility. This could benefit not only to the patient’s treatment, but also could be useful as a preventive DMD diagnostic in prenatal testing [26].

The MLPA results showed deletion fragments in three male patients, as well as a duplication that was not able to be detected with the multiplex PCR method used previously. The deletion fragments were characterized with the absence of exons 45-46-47-48-49-50-51 and 52. As DMD is an X-linked recessive disorder, these deletions clusters are easily detected in males [19] due to the total absence of dosage signal of their unique allele. The deletion clusters 45 to 50 and 45 to 52 are registered as common “hotspots” since they appear in a high frequency in previous studies [23,27]. The high density of these deletion hot spots are correlated with a high density of recombination hot spots in the same central region of the DMD gene, in the introns 44 and 50. This suggests that these two phenomena may share a common mechanism of genome instability [28]. Subsequently, the MLPA deletion
Evidence of exons 45 to 50 and 45 to 52 imply that the breaking point at the 5’ end is between exons 44 and 45, leading other investigators to search for the possible deletion junction in intron 44. Blonden and colleagues found that the most distal 80kb region of the 170kb intron 44, known as the deletion prone P20 region, is a major deletion hot spot with 113 breaking points mapped [29], and also constitutes a recombination hot spot [28]. Meanwhile, sequence analysis of introns 50 and 51 had showed the presence of repetitive AT-rich regions which are related to DNA breaking [30].

As part of the analysis of the deletions clusters found in this project, one of the aims was to map the deletion breaking points in the introns 44 and 50. Different PCR strategies were thought useful as a complementary and confirmative technique, but some difficulties were found due to the large size of the intron 44 (~170kb [29]). Several literature inconsistencies about the real size of the intron 44 made the primers design challenging. The next step of this project should be to assess the breaking point determination for the deletion exon clusters 45 to 50 and 45 to 52, including a detailed analysis of the intron 44 sequences in order to define the real size of this fragment.

The results also revealed a duplication fragment of exons 53 to 57 in one young male patient. Very few researches have been done on duplications in the DMD gene because of their low frequency (~11%) and the difficulties of detecting them with molecular methods [31]. The probe hybridization basis of the MLPA technique has overcome the challenging detection of duplications, drawing attention to the fact that the highest duplication frequency is near the 5’ end of the DMD gene, with a duplication of exon 2 being the single most common duplication [31], and the most common duplication cluster is between exons 3 to 7 [32]. A low frequency of duplication clusters in the central region of the gene, exons 53 to 55 [32] and exons 44 to 57 [33], are the closest reported duplications to the Ecuadorian duplication. The duplication of exons 53 to 57 found in one of the Ecuadorian patients, is consider as de novo mutation since the mother is not a carrier of the duplication. A previously reported duplication of exons 53 to 57 showed a germline genetic origin [24], which differs from the duplication in the Ecuadorian sample. This finding may support the hypothesis of the presence of specific mutations that affect DMD patients from Ecuador due to their mixed genetic background.

Both deletions and duplications, found in this study are related to the positive DMD phenotype of the patients. These kinds of mutations are common to be frame shift changes that affect dystrophin protein. Mutations leading to a truncated protein cause the severe phenotype of DMD, whereas mutations retaining the mRNA reading frame cause the more mild phenotype of Becker muscular dystrophy (BMD). Disruption of the translational reading
frame seems to be an important factor in deciding the severity of the disease in DMD, as compared to the extent of deletions or duplications [16].

The patients found with no deletions are not exempt of having the disease since the absence of deletions or duplications in this samples leads to the assumption that they may be affected by point mutations, which are unable to be detected by the MLPA technique. The limitation of MLPA in the screening of point mutations and intronic changes drives to the necessity of developing new molecular strategies able to diagnose 100% of Duchenne muscular dystrophy mutations with one technique. The combination of high throughput molecular techniques such as comparative genome hybridization (CGH) and next generation sequencing allows the screening of intronic changes, small deletions, specific breaking points and point mutations in addition to the common deletions and duplications in the DMD gene [34]. Nevertheless, the high cost of these technologies makes the complete diagnostic unaffordable for the patients.

Even though MLPA exhibits some limitations, the screening of the most common mutations that cause DMD is of great importance for determining a treatment strategy. The potential therapies being tested for DMD, such as exon skipping and PTC 124 commercially known as Ataluren (a novel small molecular agent design to make ribosomes less sensitive and able to ignore premature stop codons or nonsense mutations [35]), are absolutely dependant on precise knowledge of the mutation [16]. The MLPA results of this research project conclude that the most common deletions that cause Duchenne muscular dystrophy, exons 45 to 50, are present between Ecuadorian patients. In addition, this study reports a de novo duplication of exons 53 to 57, which assumes that other different mutations are also present as cause of the disease. Large studies in the Ecuadorian population may discover other DMD mutations that can be related to their mix genetic background.
ACKNOWLEDGEMENTS

This work was supported by the School of Molecular Medical Sciences and the Human Genetics Research Laboratory (Reference project number: A10767) from The University of Nottingham, with additional financial support of the SENESCYT scholarship program of the Ecuadorian government. Special acknowledgements to Dr Sally Chappel and Dr Tamar Guetta-Baranes for your encouragement, comments and accurate direction, which enhanced the quality of this research work. Total gratefulness with Dr Elizabeth Johnston and Dr Shirley Burrows, and the Molecular Genetics Service from Nottingham University Hospitals NHS Trust for the training and support. Huge thanks to the Molecular Genetics Laboratory from the Hospital de Especialidades de las Fuerzas Armadas in Ecuador, particularly to Dr Rosario Paredes M.D. and Ing Rodrigo Vinueza for providing the samples for this research project. Complete appreciation with the whole staff from the Molecular Genetics Diagnostic Laboratory from The University of Nottingham, especially with Dr Marian Hill, for allowing the Genemapper analysis of this work results.

REFERENCES


Duchenne (DMD) as compared with Becker (BMD) muscular dystrophy. *Journal of Neurological Sciences* 102: 190-196.


