The importance of early clinical diagnosis in Duchenne muscular dystrophy: mutations found in seven Ecuadorian patients

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Abstract

Context
Duchenne muscular dystrophy (DMD) is a recessive X-linked genetic disease caused by mutations in the dystrophin gene. In Ecuador, the procedure to diagnose this disease is not standardized by the public health system. The aim of this study was to propose an algorithm for DMD diagnosis in order to establish a standard protocol, emphasize early diagnosis and identify pathological mutations in affected patients.

Subjects and methods
We reported seven Ecuadorian male patients with clinical signs of DMD. They were evaluated by pediatricians, neurologists and geneticists, who made a medical record considering age of onset, pedigree, symptoms, serum CK levels and EMG analysis results. The confirmatory diagnosis and the type of mutations were identified by molecular genetic testing.

Results
The most common symptoms reported from patients were frequent falls, unstable gait, diminished muscular strength, calf pseudohypertrophy and difficulty climbing stairs. Moreover, two types of mutations in DMD gene were found, duplications and deletions. The effects of mutations in the reading frame were out-of-frame for all patients, except for one, whose mutations showed an in-frame effect on the gene.

Conclusions
It is important to emphasize the timely diagnosis of DMD to reduce the appearance of new cases, as well as the emotional impact on families. When there is a suspicion of a neuromuscular condition in male children, we recommend following the proposed algorithm in order to offer an early and efficient DMD diagnosis, confirm the disease and to provide an appropriate genetic counseling to patients and their families.

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Introduction

Duchenne muscular dystrophy (DMD, MIM #310200) is a recessive X-linked disease with a prevalence of 1 in 3,500 live born males. DMD is caused by mutations in the dystrophin gene (DMD, MIM #300377) located on chromosome Xp21. Approximately 64% of dystrophin gene mutations causing DMD are large deletions; 30% are point mutations; and 6% are duplications. This gene is one of the largest genes reported, spanning approximately 2,300 kb and consisting of 79 exons. In muscles, the dystrophin transcript (14 kb) encodes an approximately 400 kD protein. It can be found in several isoforms in distinct tissues, and is mainly expressed in the brain as well as skeletal and cardiac muscle.

The most frequent symptoms of DMD are abnormal gait at an early age, frequent falls, progressive difficulty getting up from the floor with Gower’s sign,
and pain in the muscles, especially the calves. Symptoms often begin with progressive muscle weakness at the age of three; at nine or ten the patient is non-ambulatory. The life expectancy usually does not surpass the second decade of life due to respiratory and cardiac complications.

Studies have reported that the most common algorithms used to diagnose and manage DMD include: 1) clinical evaluation; 2) pedigree chart; 3) serum creatine kinase (CK) levels; 4) electromyogram (EMG) analysis; 5) muscular biopsy (immunohistochemical analysis); and 6) genetic tests (mPCR, MLPA and sequencing). If the diagnosis is positive, female relatives of the patient are advised to be evaluated as DMD carriers, which also involves psychological treatment and reproductive planning.

In developing countries, patients with DMD face difficulties such as limited access to guidance about the disease and its management, an insufficient number of specialists in genetic disorders, high cost of diagnosis, and a lack of access to specific pharmacological treatments and disease monitoring. Moreover, a delay in confirming the correct diagnosis is a common problem, often leading the family to seek traditional treatment or complementary medicine for symptomatic relief. It may also result in the birth of a second male in the family with DMD due to the absence of genetic counseling. Furthermore, patients may develop secondary complications such as restrictive airway disease or cardiomyopathy.

In Ecuador, the procedure used to detect DMD is not standardized by the health system, causing a lack of interdisciplinary coordination between health professionals and the exclusion of many diagnostic tests. Moreover, the standard diagnostic algorithm reported by several studies is not followed in most Ecuadorian hospitals and health care centers due to limited economic and technological resources.

The present study aims to describe how seven Ecuadorian patients have been diagnosed with DMD and propose a diagnostic algorithm in order to establish a standard protocol, emphasize early diagnosis and identify pathological mutations.

Subjects and methods

This is a descriptive study about seven Ecuadorian male patients with clinical signs of DMD who were previously evaluated by pediatricians and neurologists in several medical centers of Quito-Ecuador. The patients were referred to Servicio de Genética Médica (SGM) of Hospital de Especialidades de las Fuerzas Armadas Nº1 (HE-1) to the DMD molecular diagnosis, between 2009 and 2014. SGM geneticists made a medical record for each patient considering age of onset, symptoms, pedigree, serum CK levels and EMG analysis results. The respective informed consent was signed by each patient or legal representative to approve the genetic testing.

To molecular diagnosis, a peripheral blood sample of 3 mL was collected in tubes containing the anticoagulant EDTA. Genomic DNA was isolated using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The initial molecular analysis was multiplex PCR (mPCR), which was performed by SGM in three separate reactions sets (A1, A2 and B) using a QIAGEN® Multiplex PCR Kit (Qiagen, GmbH, Hilden, Germany). The mPCR sets were optimized to detect the deletion of the following DMD gene exons: Pm, 3, 4, 6, 8, 12, 13, 17, 19, 43, 44, 45, 47, 48, 50, 51, 52 and 60. When mPCR did not detect deletions, MLPA was performed either by the Centre Hospitalier Regional Universitaire (CHRU) in Montpellier, France; or by CGC Genetics in Porto, Portugal. When mutations were identified, each one was analyzed in Leiden Muscular Dystrophy Database (LMDp, www.dmd.nl/) in order to verify the effects of mutation on dystrophyn gene reading frame and confirm the DMD diagnosis.

### Table 1. Multiplex PCR sets used to amplify 18 exons of the DMD gene, with the corresponding product size.

<table>
<thead>
<tr>
<th>SET A1</th>
<th>Total exons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exons</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>17</td>
</tr>
<tr>
<td>Product size (bp)</td>
<td>547</td>
</tr>
<tr>
<td>SET A2</td>
<td>Total exons</td>
</tr>
<tr>
<td>Exons</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>51</td>
</tr>
<tr>
<td>Product size (bp)</td>
<td>459</td>
</tr>
<tr>
<td>SET B</td>
<td>Total exons</td>
</tr>
<tr>
<td>Exons</td>
<td></td>
</tr>
<tr>
<td>Pm</td>
<td>3</td>
</tr>
<tr>
<td>Product size (bp)</td>
<td>535</td>
</tr>
</tbody>
</table>
Table 2. Clinical and molecular information compiled by SMG geneticists on the seven Ecuadorian patients with DMD

<table>
<thead>
<tr>
<th>PATIENT NUMBER</th>
<th>AGE OF QUERY</th>
<th>AGE OF ONSET</th>
<th>SYMPTOMS</th>
<th>PEDIGREE</th>
<th>SERUM CK LEVELS</th>
<th>EMG ANALYSIS</th>
<th>MOLECULAR GENETIC TESTS</th>
<th>MUTATION TYPE</th>
<th>EFFECTS OF THE MUTATIONS ON DYSTROPHIN GEN</th>
</tr>
</thead>
</table>
| 1*             | 7 years      | 4 years      | -Current falls  
- Diminished muscular strength  
- Unstable gait  
- Calf pseudohypertrophy  
- Positive Gower’s sign | - Parents non-consanguineous  
- Carrier mother | 19854 U/L | Myopathy confirmed | mPCR and MLPA | Duplication Exon 51 | Out-of frame |
| 2*             | 10 months    | ND           | -Asymptomatic | - Parents non-consanguineous  
- Carrier mother  
- Older sibling affected (Patient 1) | ND | ND | MLPA | Duplication Exon 51 | Out-of frame |
| 3**            | 7 years      | 3 years      | -Current falls  
- Calf pseudohypertrophy  
- Loss of ambulation at 7 years | - Parents non-consanguineous  
- Carrier mother  
- Non-carrier maternal grandmother | 2017 U/L | ND | mPCR | Deletions Exons 45 to 50 | Out-of frame |
| 4**            | 6 months     | ND           | -Asymptomatic | - Parents non-consanguineous  
- Carrier mother  
- Non-carrier maternal grandmother  
- Older sibling affected (Patient 3) | ND | ND | mPCR | Deletions Exons 45 to 50 | Out-of frame |
| 5              | 3 years      | 14 months    | -Progressive diminished muscular strength | - Parents non-consanguineous | 3000 U/L | ND | mPCR and MLPA | Duplications At least exons 3 to 5 | In-frame |
| 6              | 4 years      | 18 months    | -Calf pseudohypertrophy  
- Diminished muscular strength in upper and lower limbs  
- Current falls  
- Walks on tiptoes  
- Difficulty climbing stairs | - Parents non-consanguineous  
- Carrier mother | ND | ND | mPCR and MLPA | Duplication Exon 17 | Out-of frame |
| 7              | 9 years      | 4 years      | -Calf pseudohypertrophy  
- Diminished muscular strength in upper and lower limbs  
- Positive Gower’s Sign  
- Unstable gait | - Parents non-consanguineous  
- Family history of hearing loss, leukemia, diabetes and intellectual disability | ND | ND | mPCR | Deletions Exons 50 to 52 | Out-of frame |

*patient numbers 1 and 2 are siblings. **patient numbers 3 and 4 are siblings. CK: creatine kinase. EMG: Electromyogram. ND: no data
Figure 1. Algorithm for DMD diagnosis
Results

The information compiled by SGM geneticists about the seven Ecuadorian patients diagnosed with DMD is shown in Table 2. Five of the seven patients had an approximately three-year delay in diagnosis; only one patient had all of the basic diagnostic tests performed. The patients were diagnosed with DMD at the ages from three to nine, but the onset of their symptoms began between the first and third year.

The most common symptoms reported from five out of the seven patients during the physical examination were frequent falls, unstable gait, diminished muscular strength, calf pseudohypertrophy, and difficulty climbing stairs. In patient numbers one, three, and five, the serum CK levels were remarkably higher than the reference value (0-170 U/L). Patient one had a positive EMG analysis (myopathy), and the other patients indicated in the interview that they had not performed these tests; (Table 2).

Even though patient number two (ten months old) and number four (six months old) were asymptomatic for DMD, they were diagnosed directly by molecular genetic tests because their older siblings were DMD-positive (patients one and three, respectively).

Two types of mutations in DMD gene from all patients were found: (1) duplications (patient numbers one, two, five and six), and (2) deletions (patient numbers three, four and seven). The mutated exons of each patient are described in Table 2. The effects of mutations on dystrophin gene reading frame, according to LMDp, were out-of frame for all patients except for the patient number five whose mutations showed an in-frame effect on the gene.

Molecular detection of DMD female carriers (patients’ mothers) was performed in only three mothers because they were agreed with informed consent to perform the genetic tests. The mothers of patients one and two, three and four, and six were diagnosed as carriers because they showed the same mutation found in their sons respectively; (Table 2).

Discussion

Based on these findings, we identified two main problems that delay the diagnosis of DMD: (1) a diagnostic protocol has not been standardized and disseminated throughout the country, leading to varying clinical interpretations between the multidisciplinary teams of medical professionals involved in making the diagnosis; and (2) the patients are referred to genetic counseling without basic DMD tests (CK levels and/or EMG analysis) performed. To reduce the time of diagnosis, we propose an algorithm (Figure 1) which standardizes the protocol required in order to give a timely DMD diagnosis.

The proposed algorithm was structured according to Ecuadorian resources and includes the following steps: (1) clinical consultation: physical examination and pedigree chart; (2) muscular function tests: serum CK levels and EMG analysis; and (3) molecular genetic tests: mPCR, MLPA and DNA sequencing; (Figure 1).

This algorithm omits muscle biopsy but includes the most common genetic tests used to diagnose DMD. Several clinicians consider muscle biopsy unnecessary if genetics tests are performed first, particularly if the parents judge that the procedure is traumatic for the child [17]. Furthermore, a muscle biopsy must be performed according to the clinical situation, available technology and whether the differential diagnosis includes other types of muscular dystrophy in addition to DMD [18]. According to the proposed algorithm, the genetic tests that must be performed are multiplex PCR (mPCR) and multiplex ligation-dependent probe amplification (MLPA). The mPCR test was designed to amplify certain exons, but is not able to detect DMD gene duplications [19]. The MLPA test detects all deletions and duplications in the 79 DMD gene exons and allows for the identification of both affected males and female carriers [12, 15, 16].

Duplication-Cases

Patient numbers one, five, and six did not show mutations by mPCR but had evident DMD clinical signs; thus, an MLPA molecular test was performed for these patients.

Patient number one showed a duplication of exon 51. The patient had inherited this mutation from his mother, who carried the same duplication and showed high CK levels (356 U/L), which is observed in approximately 70% of female carriers [17]. The parents of this patient received an early diagnosis and genetic counseling about their son’s condition. Despite the information provided, the parents made the decision to have another child, who also inherited the disease (patient number two). The role of pediatricians, neurologists, and clinical geneticists is to provide the family guidance to understand and accept the disease, offer a health management plan for patients, and prevent new cases by giving the parents the opportunity to make informed and responsible decisions [19]. However, autonomy in decision-making is the right of the parents, and must be respected as a part of medical ethical principles [18].

Another familial case was patient number six who showed a duplication of exon 17 (Table 2). The molecular testing showed that this mutation was inherited from his mother, so she was considered female carrier.

For patients numbers one, two, and six, it was not possible to trace the origin of mutations because the grandparents were no longer living. However, according to Hu et al. (1990), germline duplications in the DMD locus appear to originate more often in males than in females, possibly due to differences in germ-cell development or the lack of homologous pairing of the DMD region in meiosis. Furthermore, the LMDp confirms that
duplications are more frequently observed in familial cases so that familial recurrence risk is increased.

Moreover, the LMDp evidenced that a duplication of the exons 51 and 17 produce an out-of frame effect on dystrophyn gene reading frame. The out-of frame mutations lead to early truncated and non-functional dystrophyn proteins and they are associated with the severe DMD phenotype[19]. The mutations found in each patient confirmed that the progressive damage in their muscular systems was associated with DMD disease.

Patient number five showed duplications of at least exons 3 to 5. According with LMDp, duplications of exons from 3 to 5 cause an in-frame effect on dystrophyn gene reading frame. In-frame mutations still allow partial production of protein, and these mutations are found in both Becker Muscular Dystrophy (BMD) and DMD patients[19], because DMD may result largely from frame shift duplications and BMD usually results from duplications that maintain the translational reading frame[20]. However, there is an exception to the in-frame duplications in exons from 3 to 4 and exons from 2 to 7 because these mutations are associated with severe and intermediate phenotypes of DMD, probably due to the structural differences of the resulting proteins[19]. According to the symptoms, the genotype of the patient and the scientific information compiled, it was confirmed the positive DMD diagnosis for patient number five.

Deletion-Cases
Patient numbers three, four, and seven were diagnosed using mPCR. Patients three and four (siblings) were diagnosed with deletions in exons 45-50, and patient seven showed deletions in exons 50-52 (Figure 2).

Oudet et al.[21] and Oshima et al.[22] confirmed that the most commonly deleted regions in the DMD gene are located between exons 45 and 52. Furthermore, LMDp evidenced that deletions in the exons 50-52 and 45-52 produce an out-of frame effect on dystrophyn gene reading frame (non-functional dystrophin protein), that confirmed the DMD diagnosis for this patients.

In the particular case of patient number three, the pediatric clinical evaluation did not validate the initial symptoms described by the mother about her first son’s gait instability. Consequently, the diagnosis was delayed and the lack of genetic counseling led to the birth of an affected sibling (patient number four).

Timely diagnosis depends on two steps: (1) the time at which first symptoms are noted; and (2) the time between symptom onset and final diagnosis. Neglecting to detect early symptoms is one of the main causes of delayed diagnosis which increases the familial recurrence risk[18]. Patients three and four
inherited their deletions from their mother, who had the same mutation and showed high CK levels (498 U/L). We suggest that the mutations in the mother were de novo because the maternal grandmother was not a carrier (MLPA negative) and the grandfather was not alive. According to Grimm et al. [8], the vast majority of deletions arise during oogenesis; consequently, there is a preferential maternal origin.

In conclusion, it is important to emphasize the timely clinical diagnosis of DMD to reduce the appearance of new cases as well as the emotional impact on families. When there is a suspicion of a neuromuscular condition (delayed walking and/or gait problems), particularly in male children, we recommend following the proposed algorithm in order to offer an early and efficient DMD diagnosis. The identification of dystrophin gene mutations and their genealogical origins is relevant to confirm the diagnosis and to provide genetic counseling to patients and their families. Finally, promoting health programs with an emphasis on genetic counseling services and clinical monitoring for patients with genetic disorders is necessary to improve the link between clinical genetic services and basic health care.

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Author contributions

The authors contributed equally in the study.

Conflict-of-interest

The authors have no conflicts of interest

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References