Review Article

Diagnostic Approaches of Duchenne Muscular Dystrophy (DMD): One Critical View

Niraj Kumar Srivastava[1,2,3*], Somnath Mukherjee[2,4] and Vivek Dixit[5]

[1]Department of Neurology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, India

[2]School of Life Sciences, Jawaharlal Nehru University, India

[3]School of Science (SOS), Indira Gandhi National Open University (IGNOU), India

[4]Bapu Nature Cure Hospital & Yogashram, Mayur Vihar Phase-1, India

[5]Department of Orthopaedics, All India Institute of Medical Sciences (AIIMS), India

ARTICLE INFO

Received Date: October 22, 2020
Accepted Date: December 24, 2020
Published Date: December 30, 2020

KEYWORDS

Duchenne muscular dystrophy
Becker muscular dystrophy
Lipid components; Metabolomics
NMR spectroscopy; Histopathology
Muscular Dystrophy
Diagnostic approaches

Citation for this article: Niraj Kumar Srivastava, Somnath Mukherjee and Vivek Dixit. Diagnostic Approaches of Duchenne Muscular Dystrophy (DMD): One Critical View. SL Clinical Medicine: Research. 2020; 3(1):119

Corresponding author:
Niraj Kumar Srivastava,
School of Science (SOS), Indira Gandhi National Open University (IGNOU), New Delhi-110068, India, Tel: +91 9650868861;
Email: nirajsuprabhat@gmail.com

ABSTRACT

Muscular dystrophy is a genetic disease and characterized by progressive muscle wasting and weakness with variable distribution and severity. Duchenne Muscular Dystrophy (DMD) is a lethal and the most rapidly progressive forms of dystrophy. Clinical symptoms in DMD patients are started in between the age of 3-5 years. These are difficulty in standing, walking, running and climbing the stairs. Frequent falls are also observed in these patients. Muscle wasting and weakness are symmetrical and selective. The major cause of DMD is mutation in the dystrophin gene, which is responsible to produce the abnormal dystrophin protein and this protein is required for the structural and functional integrity of the muscle membrane. Becker Muscular Dystrophy (BMD) has also risen due to mutations in the dystrophin gene, a milder form characterized by a slower disease course. Diagnosis of DMD/ BMD is a multistep process and this is started on the basis of clinical symptoms and signs. Two diagnostic signs [Gower's sign and Pradhan sign] are important in the diagnosis of DMD/BMD. In laboratory based diagnostic methods are electromyography (EMG), CK (Creatine Kinase) measurment in serum, multiplex PCR (polymerase chain reaction) or m PCR based gene mutation analysis, MLPA (multiplex ligation dependent probe amplification) based diagnostic method, Southern hybridization based diagnostic method and immunohistochemical based diagnostic method. Two diagnostic methods are in very early phase and these methods are metabolomics based diagnostic method and Raman hyperspectroscopy based diagnostic method. Huge work is needed to establish these two diagnostic methods for DMD / BMD diagnosis. In all these well establish method of diagnosis, the immunohistochemical based diagnostic method is the gold standard for establishing the diagnosis of DMD and BMD. This technique is clearly differentiated DMD and BMD. In all well establish methods of DMD /BMD diagnosis is based on the blood specimen except immunohistochemical based diagnostic method. This method needed muscle biopsy specimen. Procedure of muscle biopsy is invasive and painful. In this regard, clinicians want to avoid this procedure. Due to this specific reason, there is always needed to develop the blood based ultimate diagnosis for DMD/BMD patients. By considering this reason, in future, metabolomics based diagnostic method may develop as one of the ultimate

diagnostic methods for DMD/BMD, which requires the blood specimen. This method may also differentiate DMD / BMD.

ABBREVIATIONS

DMD: Duchenne Muscular Dystrophy; BMD: Becker muscular dystrophy; CK: Creatine kinase; EMG: Electromyography; TG: Triglycerides; PL: Phospholipids; CHOLest: Cholesterol ester; CHOL: Free cholesterol; FA: Fatty acids; mPCR: Multiplex polymerase chain reaction; MLPA: multiplex ligation dependent probe amplification; IHC: Immunohistochemistry; MUPs: Motor unit potentials; NMR spectroscopy: Nuclear Magnetic Resonance spectroscopy.

INTRODUCTION

Muscular dystrophy is a genetic disorder and categorized by progressive muscle wasting and weakness with variable allocation and severity. Discriminating involvement, considerable wasting and weakness of muscles is the crucial features of muscular dystrophy. At the onset of the age, progress, site of involvement and the inheritance pattern, there are numerous types of muscular dystrophy have been demonstrated in the literature. Duchenne Muscular Dystrophy (DMD) is one of the major types of muscular dystrophy. Duchenne Muscular Dystrophy (DMD) is a lethal X-linked recessive neuromuscular disorder caused by mutations in the dystrophin gene. The incidence rate of DMD is 1 in 3500 live male births [1,2]. Prevalence of DMD has been reported as 15•9 cases per 100 000 live male births in the USA and 19•5 cases per 100 000 live male births in the UK [3-5]. In India, the hospital based report showed the relative prevalence of DMD is 30% [6]. Becker Muscular Dystrophy (BMD) has also risen due to mutations in the dystrophin gene. BMD is a milder form of the disease with a later age of onset and a slower clinical progression [7].

DYSTROPHIN GENE

DMD and BMD are appearing due to mutations of the dystrophin gene [7]. The positioning of this gene is on the short arm of the X-chromosome (Xp21.2). Dystrophin protein is the product of this gene and this is a normal cytoskeleton protein of the muscle membrane. A mutation in the dystrophin gene is responsible for the production of defective dystrophin protein of muscle membrane [1-8].The dystrophin gene is a very largest human gene (0.1 % of the entire human genome) and it contains 2.4-3.0 Megabases (Mb) of DNA. The coding region of this gene contains 1 to 79 exons, which is separated by introns of 200 Kb size. The exons of this gene are represented by 14 kDa of mRNA [8,9].

Mutation in dystrophin gene

The mutation rate of dystrophin gene is exceptionally high because of its large size [10]. Numerous reports have shown mutation at the dystrophin locus of the X-chromosome in patients with DMD (severe and lethal form) and BMD (milder form) [11]. The difference between DMD and BMD explained by the reading frame hypothesis, mutations which disrupt the reading frame causing a premature termination and loss of dystrophin, is leading to a severe phenotype DMD. Mutations that retain the reading frame (in frame) generate a shortened protein; the dystrophin may still have been limited to almost normal function, leading to a milder phenotype BMD [6-8]. Simultaneously, other investigators revealed significant information pertaining to the gene and the gene product. With the application of immunoblotting technique, nonexistence of dystrophin in DMD muscles was established [13-16]. In patients with DMD, dystrophin is absent in the sarcolemma, whereas in BMD its expression is greatly reduced but is still located in the sarcolemma [17].

Pattern of mutation in dystrophin gene

Numerous mutation prototypes have been obtained in the dystrophin gene. These prototypes of mutations have been illustrated below:

Large intragenic exons deletions- On the whole 50 to 70 % of the dystrophin mutations happen for large intragenic deletions, involving several exons. The hot spot regions (major and minor) are present in the gene. The major hot spot is positioned close to the middle of the gene and encompassing exons 44 and 45. The minor hot spot envelops a broad area close to the 5' ends of the gene and numerous deletions inhabit the primary 20 exons. This is very exceptional to obtain huge deletions of additional than 60 exons [15-19]. Duplications- Dystrophin gene duplications accounts for 5-10% of all the mutations. Identification of duplication in DMD patients has been carried out by together with genomic probes and cDNA probes. On the basis of the investigation of Hu et al. [20], duplications are tandem repeats and can consequence in a genetic disorder through the interruption of exons group [20-21].

Diagnostic Approaches of Duchenne Muscular Dystrophy (DMD): One Critical View. SL Clinical Medicine: Research. 2020; 3(1):119.

Point mutation and microdeletions- Point mutations and small deletions or microdeletions were discovered at a close to the initial phase. Due to the large size and complexity of the dystrophin gene, there was a challenge for the identification of point mutations or microdeletions in the dystrophin gene. The first nonsense mutation was described by Bulman et al. [23] in exon 26 of a DMD patient, where an immunological investigation of the truncated dystrophin from muscle biopsy specimen endorsed preceding localization of the mutation. Detection of point mutation became simpler following Single-Strand Conformation Polymorphism Analysis (SSCP), the protein truncation test, hetroduplex analysis and other accessible analytical approaches [22-24].

DYSTROPHIN PROTEIN

The Dystrophin gene produces the dystrophin protein with a molecular weight of 427 kDa and possesses 3685 amino acids. This protein is localized in skeletal muscle (0.002 % of the whole muscle protein) and also obtained in heart, brain & smooth muscle with a molecular weight of 405 kDa. Identification of four domains of dystrophin protein is established. In these entire four domain, the first domain is the actin-binding N-terminal, the second domain is the rod, the third domain is the cysteine-rich and the fourth domain is the C-terminal [1,2,17-19,25]. Exons associated with these domains are described below:

Actin-binding domain (exons 1-8): The close similitude of 240 N-terminal amino acids of dystrophin to the actin-binding domains of spectrin and α-actinin expected that dystrophin would attach to actin filaments.

Rod or triple-helical-repeat domain (exons 9-62): Subsequently and biggest dystrophin domain has a mass of 300 kDa and 125 nm in length. This is encoded by exons 9 to 62 and contains 2400 amino acid residues.

Cysteine-rich domain (exons 63 to 69): This domain has 15 cysteine residues and a stretch of 142 residues that show 24 % homology to the C –terminus of α-actinin. C-terminal domain (exons 70 to 79): The fourth domain encompasses the last 420 amino acid residues of dystrophin and is highly conserved across species [1,2, 6-8, 26,27].

CLINICAL SYMPTOMS AND SIGNS

In DMD patients, the preliminary symptoms such as delayed walking, frequent falls and difficulty in running and climbing stairs are recognized between the ages of 1 and 3 years. In these patients, the muscles in the region of the calf, pelvis and thigh frequently affected first and visualizing markedly bulkier as compared to normal. DMD patients usually required a wheelchair by the age of 8–14 years. Such event is happening due to progressive muscle wasting and weakening, and ultimate consequences appeared in the form of ambulation failure. Once a patient becomes wheelchair bound, certain complications develop extra speedily including scoliosis and muscular contractures. Scoliosis, which is responsible for the spine to curve slanting and/or forward or backward, directs to extra orthopaedic troubles as one shoulder or hip becomes superior as compared to the other leading to potential respiratory problems as the chest cavity decreases. Symptoms of cardiomyopathy in the late teens can come into view in DMD patients, even though the disorder in this organ has expected to develop previously. Cardiomyopathy is responsible for enlargement of the heart's chambers and the walls to acquire thinner and in the late-teens or early 20s the situation is connected with breathing exertion and once the heart and respiratory muscles are severely wasted the situation becomes life-threatening. Even in presence of sophisticated medical facilities, patients with DMD die due to cardiac or respiratory collapse before or during their 30s [3,28,29]. Becker Muscular Dystrophy (BMD) is clinically analogous to DMD, but is a less severe form of myopathy, affecting 1/30,000 males. Patients with BMD begin to demonstrate clinical signs between 2 and 20 years of age. After comparison with DMD, progression in BMD is slower. Few patients with BMD show clinical signs comparable to those of DMD, whereas few patients are still able to walk at the age of 60 years. Incapability to walk overcomes at about 30 years old, and death is repeatedly present 30 years after the manifestation of the first clinical signs. Cardiomyopathy frequently happens in 73% of BMD patients more than 40 years age [17].

DIAGNOSTIC SIGNS

Two imperative clinical signs are supportive for the diagnostic rationale. These are Gower's sign (Figure 1) and "Pradhan sign" (Figure 2) or "valley sign". Gower's sign is appeared due

to weakness of the knees and hip extensors. This sign is visualized in DMD patient by acquiring a specific posture, when the child's efforts to go up from the floor. The another important clinical sign “Pradhan sign” or “valley sign” is visualized in patients with DMD as a linear groove or sometimes an oval depression due to the wasting of the muscles participating in the formation of the posterior axillary fold, the teris major, teris minor, posterior-most part of the deltoid and the lateral one third of the infraspinatus. On either side of the depression, two prominent mounts are noticeable, the inferomedial formed by the hypertrophied infraspinatus muscle and the superolateral by the hypertrophied deltoid muscle. The entire appearance is similar to a “valley between two mounts” [30-32]. Gower’s sign and Pradhan’s valley sign also appear in BMD [33]. In this way, these two diagnostic signs are not able to differentiate both DMD and BMD.

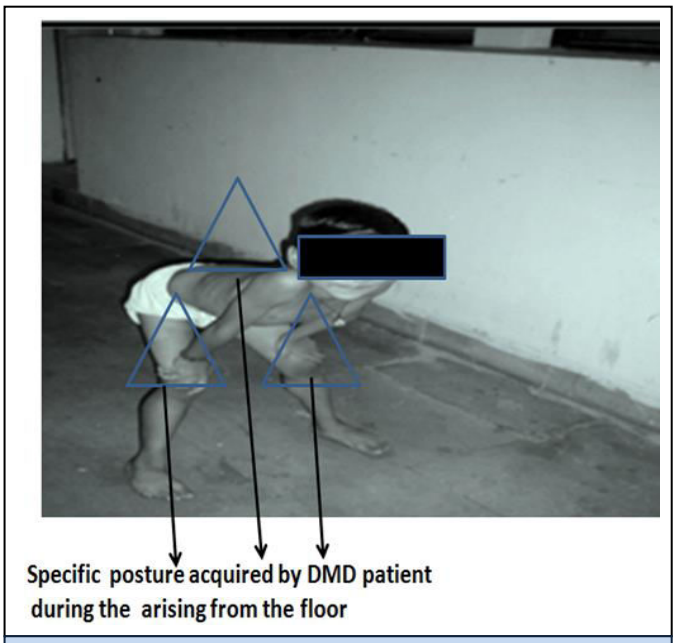

Figure 1: Gower's sign is appeared in DMD Patient during the rising from the floor.

LABORATORY BASED DIAGNOSTIC METHODS

Electromyography (EMG) or Electromyographical (EMG) Examination

Electromyography (EMG) is a laboratory assessment, which refers to methods of studying the electrical activity of muscle. EMG examination is carried out for authentication the diagnosis of myopathy in DMD patients by applying the concentric bipolar needle [33]. Needle EMG investigation is the most revealing in myopathic diseases. It can authenticate the occurrence of a myopathy, constricted down the differential, and recognize a suitable biopsy location. The number and position of muscles investigations are depends on the prototype of weakness. At least, the investigators suggested for investigating one proximal and one distal muscle from one upper extremity and from one lower extremity as well as the thoracic paraspinals. Such investigation may be enough when there is a high clinical suspicion of myopathy. In general, the investigated muscles contain the deltoid, biceps, triceps, pronator teres, extensor digitorum communis, first dorsal interosseous, gluteal muscles, iliopsoas, vasti, tibialis anterior, and gastrocnemius. The selections of extra muscles are depending on a patient's prototype of weakness and the clinical doubt [33-36].

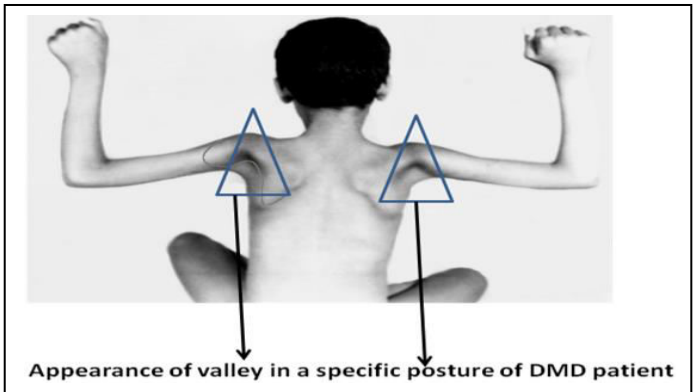

Figure 2: Valley sign is appeared in DMD patient due to muscle wasting.

Investigation of Motor Unit Action Potential (MUAP) morphology and recruitment pattern is the key constituent of needle EMG that facilitates to find the diagnosis of a myopathy. In myopathic procedures, there is a failure or dysfunction of entity muscle fibers. In this regard, there is a marked reduction in the size of the motor unit. The number of accessible motor units does not modify since the pathologic procedure happens distal to the motor axons. These consequences are raised in the emergence of short, small, polyphasic MUAPs. Myopathy is well established following the appearance of the myopathic EMG pattern. The myopathic EMG pattern appeared in the form of predominately small amplitude (200 µv per division) and short duration (10 ms/cm) motor units potential (MUPs) to enhance in the proportion of polyphasia (Figure 3) [36-38]. EMG pattern is also similar in patients with BMD [33-38].

Diagnostic Approaches of Duchenne Muscular Dystrophy (DMD): One Critical View. SL Clinical Medicine: Research. 2020; 3(1):119.

Measurement of serum CK (Creatine Kinase)

The worth of serum CK (creatine kinase) has been paid to its diagnostic assist in clinical medicine. The enzyme is obtained in elevated concentrations in skeletal and cardiac muscle and a slighter degree in the brain tissue, however, is only obtained in the comparatively little concentration in other tissues [39,40]. The distinctive observation in DMD is a noticeably elevated serum CK level, at least 10 to 20 times (and often 50 to 200 times) the upper limit of normal before the age of five years. Serum CK concentrations are also raised even in newborns and preceding to any symptoms. The high CK levels at birth can establish the basis of neonatal screening for DMD. Levels are reaching a peak at two to three years of age and then decline with progressing age as well as progressive wasting of dystrophic muscle fibers [39-41]. Serum CK measurement is a very simple diagnostic tool for DMD patients and average CK value is found to be 9387.51 IU/L. The minimum and maximum values of CK has been estimated 1183 IU/L and 29 000 IU/L, respectively [42]. In both DMD and BMD serum levels are usually very high (50 to 100 times the normal serum level). Serum level is particularly elevated early in the disease and may drop with disease progression [1,2, 30,31, 39-41]. A measurement of serum CK level is also not capable of differentiation in DMD and BMD.

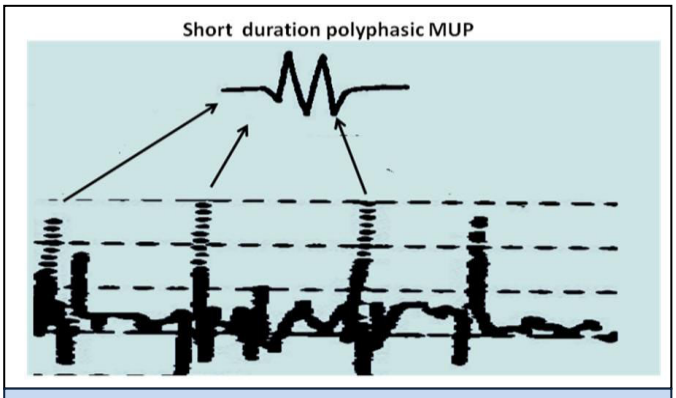

Figure 3: Myopathic EMG pattern in DMD (abnormal EMG pattern).

Multiplex PCR (mPCR) based diagnostic method

Multiplex PCR (mPCR) is a frequent and well established approach to the DMD/ BMD diagnosis in India. The mPCR approach is predominantly qualitative or semi-quantitative and this allows the revealing of approximately 98 % of entire the deletions, which envelops the recognition of the entire the 65% mutations [42,43]. Isolation of DNA was carried out from peripheral blood leukocytes with the application of standard phenol/chloroform methods. Amplification of multiplex DNA of the dystrophin gene was performed by the use of the Chamberlain and Beggs primers. By applying two multiplex PCR assays, permitting the amplification of 9 exons each: the Chamberlain reaction using primers for exons 45, 48, 19, 17, 51, 8, 12, 44, 4, and the Beggs reaction performed with primers for the promoter and exons 3, 43, 50, 13, 6, 47, 60, and 52 [44]. After completing the process of amplification, the PCR products were separated on 2% agarose minigels and the bands visualized by staining with ethidium bromide (Figure 4) [43]. mPCR based diagnostic method is not capable of differentiation in DMD and BMD.

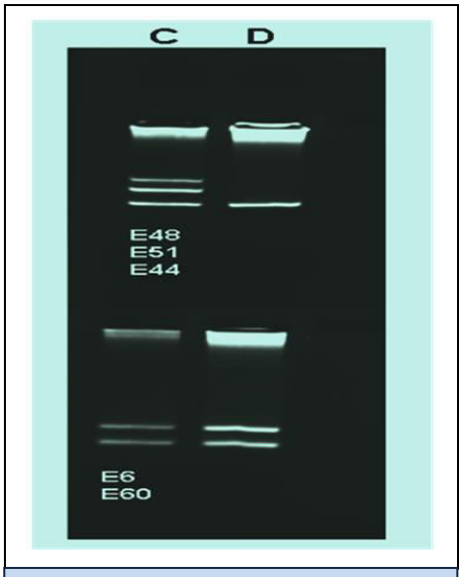

Figure 4: Multiplex PCR based electrophoretic pattern showed the deletion in exons 48 & 51 in DMD patient [C =Control; D= DMD patient].

MLPA (Multiplex Ligation Dependent Probe Amplification) based diagnostic method

MLPA (Multiplex Ligation Dependent Probe Amplification) has facilitated more consistent and faster quantitative deletion of the entire dystrophin gene includes 79 exons to investigate the deletions and duplications. Subsequent multiplex PCR by MLPA enhances the percentage of patients (DMD/ BMD) with a defined diagnosis to 75 %. MLPA-based diagnostic method is applied to diagnose DMD/ BMD in USA, China and several European countries [43]. A more recently develop approach is

Multiplex Ligation-Dependent Probe Amplification (MLPA), which has been found to afford cost-effective, reliable, and rapid screening of multiple loci for copy number changes. In MLPA based investigation, two sequence-tagged half-probes (one small, synthetic half-probe and one large, M13-derived half-probe) are hybridized to their genomic target sequence and ligated together at 54°C using thermostable DNA ligase. Amplification of the ligated probes is carried out and then amplified by fluorescently labeled universal primers that correspond to the probes' sequence tags. Every probe is designed to be uniquely sized, resulting in a ladder of amplified products that can be visualized and quantified by automated fluorescent electrophoretic analysis. In the current situation, MLPA can screen at least 40 loci in one reaction with as little as 20 ng template DNA [45]. The MLPA based diagnostic method is also not capable of differentiation in DMD and BMD.

Southern hybridization based diagnostic method

The investigation of gene mutations has significantly enhanced diagnosis, carrier detection, and genetic counseling of patients with DMD/ BMD. With the capability to recognize deletions and duplications in ~70% of affected patients, accurate direct DNA testing can be applied in these cases. By applying full-length dystrophin cDNA clones to probe Southern blots, there is a possibility of directly detect deletions and duplications. The cDNA probes identify the location of the mutation itself, so meiotic recombination actions are irrelevant. In this regards, the probability of diagnostic error is deeply minimized. The digested and blotted DNA is in succession hybridized with seven to nine cDNA probes, which wrap the complete 14-kb transcript. Just about 10 exons are scored for hybridization of every cDNA. Nevertheless, the deletions are chiefly located in two hotspots; in this way, the majority of deletions can be well recognized by four cDNA probe hybridizations. The deletions are basically detected by investigation of Southern blots for the presence or absence of every exon includes genomic restriction fragments that hybridize to the cDNA probe. A male control is incorporated on all Southern blots to demonstrate the appropriate position and intensity of the restriction fragments. Duplication is exposed by an amplified hybridization intensity of one or more DNA fragments when compared to the male control. Duplications should always be confirmed by applying a second dissimilar restriction enzyme digestion, and the autoradiogram should be scanned by densitometry. The majority of frequently used restriction enzyme for dystrophin gene mutation analysis is HindIII because the restriction prototype for all 79 exons is identified and the majority of exons are on single fragments. BglII and EcoRI are also usually used enzymes [46] (Figure 5). Southern hybridization based diagnostic method is also not capable of differentiation in DMD and BMD.

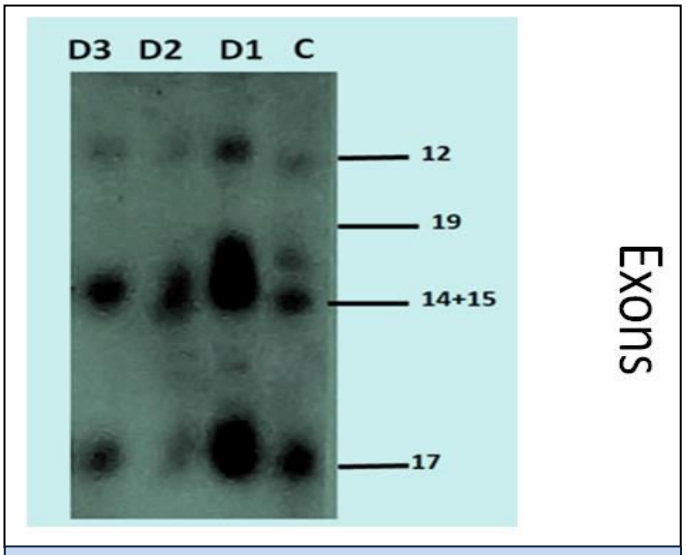

Figure 5: Analysis of gene mutation in DMD patients has been performed by Southern hybridization, showed the deletion of exon 19 in D3, D2 and D1 patients with DMD, Whereas C repersents the normal Subjects.

Immunohistochemical or immunocytochemical based diagnostic method

Immunohistochemical or Immunocytochemical based investigation of dystrophin protein in patients with DMD and BMD is performed on muscle biopsy specimen. The dystrophin is positioned at the plasma membrane of skeletal muscle fibers and established by a variety of immunohistochemical or immunocytochemical techniques. The monoclonal antibodies of dystrophin have been produced that recognize epitopes within several domains (N-terminal, rod domain and C-terminal). Antibodies against dystrophin- associated proteins (such as spectrin, α-sarcoglycan and β-dystroglycan) are also applied in this technique to carry out the finding of these proteins [47,48]. Complete or virtually complete absence of dystrophin is diagnostic of DMD while patchy preservation or partial presence of dystrophin would suggest BMD [47,48] (Figure 6).

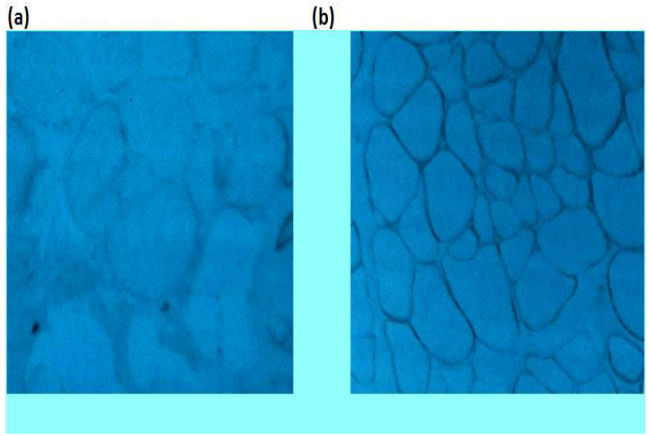

Figure 6: Dystrophin staining showed (a) the complete absence of dystrophin in the DMD patients, (b) reduced and discontinuous dystrophin in the BMD patient.

This is the gold standard for establishing the diagnosis of DMD and BMD [30,31]. This technique clearly differentiates DMD and BMD.

Metabolomics based diagnostic method

Metabolomics covers the qualitative and quantitative investigation of all the metabolites in an organism. In this way, the chief input of metabolomics in the biological sciences is the generation of the metabolic fingerprinting of an organism. Here, the further question arises, what is the metabolites and how can we define it. Metabolites are those molecules, which molecular weight is less than 1000 daltons (Da) [49-52].

Metabolomics based investigations are performed by two analytical tools. These are NMR (Nuclear Magnetic Resonance) and Mass Spectroscopy (MS). These two techniques have several advantages and disadvantages. NMR spectroscopy based metabolomics technique does not need on the separation of the analytes. This shows that the sample is not destroyed. One specific advantage of this investigation is the simultaneous measurement of all types of metabolites. Investigation of sample is quick, vigorous, enabling and high-throughput. The sensitivity of this technique is less as compared to MS techniques [52,53].

Applications of metabolomics include disease diagnosis, monitoring the effects of medical interventions including drugs, detection of adulteration of food, and analysis of biochemical pathways and their perturbations resulting from mutations, aging, diet, exercise, or life style [54]. Proton NMR spectroscopy based metabolomics approach (1H-NMR spectroscopy) has been extensively applied for clinical purposes such as in inherited disorders of lipid metabolism: SLOS, Cerebrotendinous Xanthomatosis (CTX), sitosterolemia and Refsum disease through the analysis of whole or lipid extracts of blood and plasma [42,43]. With the visualization of this application, NMR spectroscopy based lipid analysis has been also applied in DMD patients for the diagnostic purpose. Lipid constituents [Triglycerides (TG), Phospholipids (PL), Free Cholesterol (CHOL) and Cholesterol Ester (CHOLest)] found significantly elevated in serum of DMD as compared to the normal subject (Figure 7). It has been also found significantly higher level of all the lipid constituents in serum of the gene deletion positive as well as negative cases of DMD as compared to healthy individuals [42]. This technique is under developmental stage and not well established for diagnosis of DMD as well as in the differentiation of DMD and BMD.

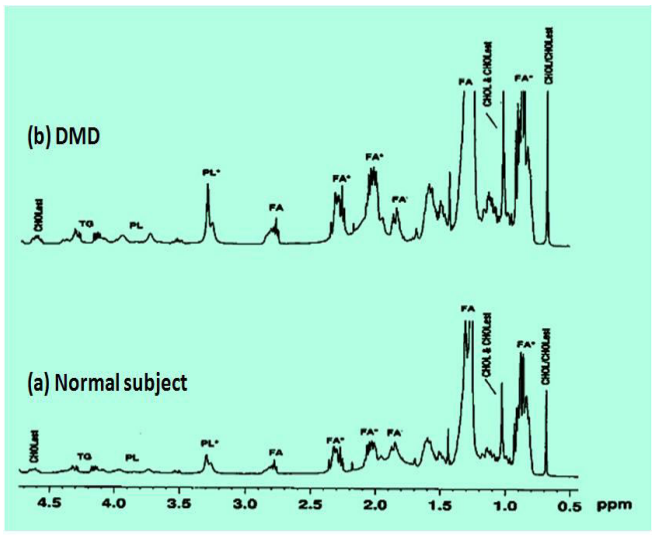

Figure 7: Proton NMR spectroscopy based analysis of lipid components in serum of DMD patient as well as in normal subject showed the elevation of lipid compnents in DMD patients [TG: Triglyceride; PL: Phospholipids; FA: Fatty Acids; CHO:: Free Cholesterol; CHOlest: Cholesterolester].

Raman hyperspectroscopy based diagnostic method

In the Raman hyperspectroscopy based investigation, the blood or serum of a mouse model of DMD was analyzed. Partial least squares discriminant analysis was applied to the spectral dataset acquired from the blood serum of a mouse model of Duchenne muscular dystrophy (mdx) and control mice. Cross validation showed 95.2% sensitivity and 94.6% specificity for identifying diseased spectra. These outcomes were verified by

external validation with 100% successful classification accuracy. These outcomes present Raman hyperspectroscopy based analysis of blood serum as an easy, fast, non-expensive, and minimally invasive detection method for distinguishing control and mdx mice model, with a strong potential for clinical diagnosis of DMD [55]. This technique is under research stage and not well established for diagnosis of DMD as well as in the differentiation of DMD and BMD.

DISCUSSION

Two important clinical signs [Gower's sign and Pradhan sign] appeared in both DMD BMD patients [30-33]. EMG pattern only demonstrates the myopathic pattern in DMD/ BMD, whereas CK estimation showed the indication of muscle disease. But, the specific pattern of rising to the level of CK in DMD/ BMD patient, represents its diagnostic importance for DMD/BMD patients [30,31,34-41]. But, differentiation in between DMD and DMD is not possible by diagnostic signs, EMG and serum CK measurement. Deletions account for about two-thirds of the mutations in dystrophin gene and mPCR allows detection of 98% of those deletions. This technique detects large deletions in about 60–65% patients, has largely replaced biopsy, and has become the preferred method of diagnosis of DMD/ BMD in many developing countries like India. However, it is qualitative and does not detect duplications, which account for 6% of mutations in dystrophin gene. Multiplex Ligation-Dependant Probe Amplification (MLPA), reliable quantitative method to detect deletions and duplications in all 79 exons of the dystrophin gene and also carrier testing. MLPA adds another 10–15% positive case of DMD /BMD to mPCR. Many neurologists, particularly in India, still perform muscle biopsies for the immunohistochemical or immunocytochemical based diagnosis of DMD/ BMD, and this should be performed only after available genetic testing is negative for the mutation [56]. But, differentiation in between DMD and DMD is not possible by mPCR and MLPA.

In another observation, one hypothesis have been developed that multiplex PCR (mPCR) and Southern blotting complementary to each other. There are several reasons for the establishment of this concept. Identification of duplications in DMD/BMD is performed by standard multiplex conditions and ethidium bromide staining is technically complicated because it is during the exponential phase that the amount of amplified products is proportional to the abundance of starting DNA. This occurs when the primers, nucleotides, and Taq polymerase are in large excess over that of the template concentration. After the completion of a sufficient number of cycles to visualize the PCR products on an ethidium bromide-stained gel, the PCR reaction is no longer in the exponential quantitative range and the duplicated exons appear little or no brighter as compared to the normal single copy exons. The detection of duplications with Southern blotting is relatively uncomplicated with the application of densitometry and multiple restriction digests. Nevertheless, the current application of automated DNA fragment analysis using multiplex PCR with fluorescently labeled primers has permitted extra accurate detection of duplications. Southern blotting allows determination of all deletion and duplication endpoints, which is important in determining the effect of the mutation on the reading frame. Because the majority of labs tend to assess 20 to 25 deletion prone exons by multiplex PCR, it is not possible to obtain all endpoints by PCR alone. In this way, the Southern blot technique permits for the detection of junction bands [1,2,7,30,31,46]. Now, like mPCR, clear differentiation of DMD and BMD is also not possible by Southern blotting or Southern hybridization.

Detection of point mutation and small deletions or micro deletions are not carried out by all the methods or approaches of clinical set up such as mPCR, MLPA and Southern hybridization. All these detections are confirmed by Single-Strand Conformation Polymorphism Analysis (SSCP), the protein truncation test, hetroduplex analysis and other accessible analytical approaches. All these methods or approaches are time consuming and not feasible in clinical practice [22-24,42]. All these detection approaches are used for research purposes [1,2,22-24,30]. In this way, all the analytical approaches or methods (mPCR, MLPA and Southern hybridization) in clinical set up are not applicable to detect all types of mutations in DMD/BMD. In all these well establish method of diagnosis, the immunohistochemical based diagnostic method is the gold standard for establishing the diagnosis of DMD and BMD [1-2,30,31]. This technique is clearly differentiated DMD and BMD.

In all well establish methods of DMD /BMD diagnosis is based on the blood specimen except immunohistochemical based diagnostic method. This method needed muscle biopsy specimen. A muscle biopsy is an invasive procedure and clinicians do not want to suggest for performing this procedure. But, after negative results of all types of blood based diagnostic methods, the ultimate diagnosis of DMD/BMD is carried out by the immunohistochemical or immunocytochemical based method [1,2,30,31,42,48]. So, there is a strong need for the development of blood based diagnostic method for the replacement of immunohistochemical or immunocytochemical based diagnostic method.

Proton NMR spectroscopy based metabolomics based analysis revealed that the quantitative comparison of serum lipid components (triglycerides, phospholipids, free cholesterol, cholesterol ester and total cholesterol) showed no significant difference between positive and negative gene deletion cases of DMD patients. NMR-based analysis of lipid constituents of the serum may cross the present barrier of gene mutation analysis and provide an efficient alternative diagnostic method for DMD diagnosis [42]. Similarly, Raman hyperspectroscopy based analysis of blood serum of the mdx mouse model showed the specific pattern and clearly distinguish from normal mice. So, this technique has a strong potential for clinical diagnosis of DMD [55].

NMR spectroscopy based metabolomics approach and Raman hyperspectroscopy based methods are not well established and these are blood based diagnostic methods [42,55]. Huge work is needed for the establishment of these two methods for the diagnosis of DMD/BMD as well as differentiation between them. In future, these two techniques may be established as diagnostic methods for avoiding the muscle biopsy.

ACKNOWLEDGEMENTS

The authors wish to thank Council of Scientific & Industrial Research [No.13 (8660-A) /2013-Pool] and University Grant Commission [No.F.4-2/2006 (BSR) / 13-194/2008 (BSR)], Government of India, for their generous financial support. Authors sincerely thanks to the staff of the neurophysiology laboratory for providing help in collecting electromyographical data. Senior residents are acknowledged for providing clinical data and collection of tissue specimens. Professor Rajkumar (Department of neurosurgery, SGPGIMS, Lucknow) is acknowledged for providing the normal muscle specimens.

REFERENCES

1. Emery AEH. (2000). Muscular Dystrophy: The Facts. England: Oxford University Press.
2. Emery AEH. (1998). Neuromuscular Disorders: Clinical and Molecular Genetics. First ed. Wiley.
3. Ryder S, Leadley RM, Armstrong N, Westwood M, de Kock S, et al. (2017). The burden, epidemiology, costs and treatment for Duchenne muscular dystrophy: an evidence review. Orphanet J Rare Dis. 12: 79.
4. Mendell JR, Shilling C, Leslie ND, Flanigan KM, al-Dahhak R, et al. (2012). Evidence-based path to newborn screening for Duchennemuscular dystrophy. Ann Neurol. 71: 304-313.
5. Moat SJ, Bradley DM, Salmon R, Clarke A, Hartley L. (2013). Newborn bloodspot screening for Duchenne muscular dystrophy: 21 years' experience in Wales (UK).Eur J Hum Genet. 21: 1049-1053.
6. Khadilkar S. (2008). Bird's eye view of myopathies in India. Neurol India. 56:225-228.
7. Freund AA, Scola RH, Arndt RC, Lorenzoni PJ, Kay CK, et al. (2007). Duchenne and Becker muscular dystrophy: a molecular and immunohistochemical approach. ArqNeuropsiquiatr. 65:73-76.
8. Pozzoli U, Elgar G, Cagliani R, Riva L, Comi GP, et al. (2003). Comparative analysis of vertebrate dystrophin loci indicate intron gigantism as a common feature. Genome Res. 13:764-772.
9. Lee BL, Nam SH, Lee JH, Ki CS, Lee M, et al. (2012). Genetic analysis of dystrophin gene for affected male and female carriers with Duchenne/Becker muscular dystrophy in Korea. J Korean Med Sci. 27:274-280.
10. Bennett RR, den Dunnen J, O'Brien KF, Darras BT, Kunkel LM. (2001). Detection of mutations in the dystrophin gene via automated DHPLC screening and direct sequencing. BMC Genet. 2:17.
11. Le Rumeur E. (2015). Dystrophin and the two related genetic diseases, Duchenne and Becker muscular dystrophies. Bosn J Basic Med Sci. 15:14-20.
12. Monaco AP, Neve RL, Colletti-Feener C, Bertelson CJ, Kurnit DM, et al. (1986). Isolation of candidate cDNAs for

portions of the Duchenne muscular dystrophy gene. Nature. 323:646-650.

13. Kunkel LM, Monaco AP, Middlesworth W, Ochs HD, Latt SA. (1985). Specific cloning of DNA fragments absent from the DNA of a male patient with an X chromosome deletion. ProcNatlAcadSci USA. 82:4778-4782.
14. Ray PN, Belfall B, Duff C, Logan C, Kean V, et al. (1986). Cloning of the breakpoint of an X;21translocation associated with Duchenne muscular dystrophy. Nature. 318:672-675.
15. Worton RG, Thompson MW. (1988). Genetics of Duchenne muscular dystrophy. Annu Rev Genet. 22:601-629.
16. Hoffman EP, Brown RH, Kunkel LM. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell. 51:919-928.
17. Cruz Guzmán Odel R, Chávez García AL, Rodríguez-Cruz M. (2012). Muscular dystrophies at different ages: metabolic and endocrine alterations. Int J Endocrinol. 2012:485376.
18. Danieli GA, Mioni F, Müller CR, Vitiello L, Mostacciuolo ML, et al. (1993). Patterns of deletions of the dystrophin gene in different European populations. Hum Genet. 91:342-346.
19. Worton RG, Thompson MW. (1988). Genetics of Duchenne muscular dystrophy. Annu Rev Genet. 22:601-629.
20. Hu XY, Burghes AH, Ray PN, Thompson MW, Murphy EG, et al. (1988). Partial gene duplication in Duchenne and Becker muscular dystrophies. J Med Genet. 25:369-376.
21. Hu XY, Ray PN, Murphy EG, Thompson MW, Worton RG. (1990). Duplicational mutation at the Duchenne muscular dystrophy locus: its frequency, distribution, origin, and phenotypegenotype correlation. Am J Hum Genet. 46:682-695.
22. Kodaira M, Hiyama K, Karakawa T, Kameo H, Satoh C. (1993). Duplication detection in Japanese Duchenne muscular dystrophy patients and identification of carriers with partial gene deletions using pulsed-field gel electrophoresis. Hum Genet. 92:237-243.
23. Bulman DE, Gangopadhyay SB, Bebchuck KG, Worton RG, Ray PN. (1991). Point mutation in the human dystrophin gene: identification through western blot analysis. Genomics. 10:457-460.
24. Roberts RG, Bobrow M, Bentley DR. (1992). Point mutations in the dystrophin gene. ProcNatlAcadSci USA. 89:2331-2335.
25. Brown KJ, Marathi R, Fiorillo AA, Ciccimaro EF, Sharma S, et al. (2012). Accurate Quantitation of Dystrophin Protein in Human Skeletal Muscle Using Mass Spectrometry. J Bioanal Biomed. 7:001.
26. Koenig M, Monaco AP, Kunkel LM. (1988). The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. Cell. 53:219-228.
27. Brown SC, Brown SS, Lucy JA. (1997). Dystrophin: Gene, Protein and Cell Biology. Cambridge University Press.
28. Magri F, Govoni A, D'Angelo MG, Del Bo R, Ghezzi S, et al. (2011). Genotype and phenotype characterization in a large dystrophinopathic cohort with extended follow-up. J Neurol. 258: 1610-1623.
29. Davidson ZE, Ryan MM, Kornberg AJ, Sinclair K, Cairns A, et al. (2014). Observations of body mass index in Duchenne muscular dystrophy: a longitudinal study. Eur J ClinNutr. 68: 892-897.
30. Amato AA, Russell JA.(2008). Neuromuscular Disorders. First ed. McGraw-Hill Professional.
31. Emery AEH. (2002).The muscular dystrophies. Lancet. 359:687-695.
32. Pradhan S. (1994). New clinical sign in Duchenne muscular dystrophy. Pediatr Neurol. 11:298-300.
33. Pradhan S. (2004). Valley sign in Becker muscular dystrophy and outliers of Duchenne and Becker muscular dystrophy. Neurol India. 52:203-205.
34. Mishra UK, Kalita J. (2006). Clinical Neurophysiology. Second ed. Elsevier.
35. Petajan JH. (1991). Motor unit recruitment. Muscle Nerve. 14: 489-502.
36. Daube JR. (1991). Needle examination in clinical electromyography. Muscle Nerve. 14: 685-700.
37. DeLucchi MR. (1976). Techniques and Methods of Data Acquisition of EEG and EMG. Elsevier.
38. Paganoni S, Amato A. (2013). Electrodiagnostic evaluation of myopathies. Physical medicine and rehabilitation clinics of North America 24: 193-207.

39. Colombo JP, Richterich R, Rossi E. (1962). Serum creatine phosphokinase: determination and diagnostic significance. Klin Wochenschr. 40:37-44.
40. Bushby KMD, Anderson LVB. (2001). Muscular Dystrophy: Methods and Protocols. Humana Press .
41. Yiu EM, Kornberg AJ. (2008). Duchenne muscular dystrophy. Neurol India. 56:236-47.
42. Srivastava NK, Pradhan S, Mittal B, Gowda GA. (2010). High resolution NMR based analysis of serum lipids in Duchenne muscular dystrophy patients and its possible diagnostic significance. NMR Biomed. 23: 13-22.
43. Srivastava NK, Mukherjee S, Sinha N. (2016). Alteration of phospholipids in the blood of patients with Duchenne muscular dystrophy (DMD): in vitro, high resolution 31P NMR-based study. Acta neurologica Belgica. 116: 573–581.
44. Sbiti A, El Kerch F, Sefiani A. (2002). Analysis of Dystrophin Gene Deletions by Multiplex PCR in Moroccan Patients. Journal of biomedicine & biotechnology. 2: 158–160.
45. Stern RF, Roberts RG, Mann K, Yau SC, Berg J, et al. (2004). Multiplex ligation-dependent probe amplification using a completely synthetic probe set. BioTechniques. 37: 399–405.
46. Prior TW, Bridgeman SJ. (2005). Experience and strategy for the molecular testing of Duchenne muscular dystrophy. The Journal of molecular diagnostics : JMD. 7: 317–326.
47. Vogel H, Zamecnik J. (2005). Diagnostic immunohistology of muscle diseases. J Neuropathol Exp Neurol. 64 : 181-193.
48. Dubowitz V, Sewry CA, Lane RJM. (2007). Muscle Biopsy: A Practical Approach. Third ed. Elsevier Health Sciences.
49. Beckonert O, Keun HC, Ebbels TMD, Bundy JG, Holmes E, et al. (2007). Metabolic profiling, metabolomic and metabonomic procedures for NMRspectroscopy of urine, plasma, serum and tissue extracts. Nature Protocols. 2: 2692-2703.
50. Fillet M, Frédérich M. (2015). The emergence of metabolomics as a key discipline in the drug discovery process. Drug Discov Today Technol. 13: 19-24.
51. Kosmides AK, Kamisoglu K, Calvano SE, Corbett SA, Androulakis IP. (2013). Metabolomic fingerprinting: challenges and opportunities. Critical reviews in biomedical engineering. 41: 205–221.
52. Emwas AH, Roy R, McKay RT, Tenori L, Saccenti E, et al. (2019). NMR Spectroscopy for Metabolomics Research. Metabolites. 9: E123.
53. Mirsaeidi M, Banoei MM, Winston BW, Schraufnagel DE. (2015). Metabolomics: Applications and Promise in Mycobacterial Disease. Annals of the American Thoracic Society. 12: 1278–1287.
54. Markley JL, Brüschweiler R, Edison AS, Eghbalnia HR, Powers R, et al. (2017). The future of NMR-based metabolomics. Current opinion in biotechnology. 43: 34–40.
55. Ralbovsky NM, Dey P, Galfano A, Dey BK, Lednev IK. (2020). Diagnosis of a model of Duchenne muscular dystrophy in blood serum of mdx mice using Raman hyperspectroscopy. Scientific reports. 10: 11734.
56. Manjunath M, Kiran P, Preethish-Kumar V, Nalini A, Singh RJ, et al. (2015). A comparative study of mPCR, MLPA, and muscle biopsy results in a cohort of children with Duchenne muscular dystrophy: a first study. Neurology India. 63: 58–62.