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A multi-center comparison of diagnostic methods for the biochemical evaluation of suspected mitochondrial disorders

R.J.T. Rodenburg [a,*], G.C. Schoonderwoerd [b], V. Tiranti [c], R.W. Taylor [d], A. Rötig [e], L. Valente [c], F. Invernizzi [c], D. Chretien [e], L. He [d,e], G.P.B.M. Backx [a], K.J.G.M. Janssen [a], P.F. Chinnery [d], H.J. Smeets [f], I.F. de Coo [g,1], L.P. van den Heuvel [a,h,1]

[a] 774 Nijmegen Centre for Mitochondrial Disorders, Department of Pediatrics, Department of Laboratory Medicine, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands
[b] Department of Clinical Genetics, Erasmus MC, Dr. Molewaterplein 10, 3015 GE Rotterdam, The Netherlands
[c] Division of Molecular Neurogenetics, Pierfranco and Luisa Mariani Center for the Study of Mitochondrial Disorders of Infancy and Childhood, IRCCS Foundation Neurological Institute C. Besta, Milan, Italy
[d] Newcastle Mitochondrial NSCT Diagnostic Laboratory, Newcastle University, Newcastle upon Tyne, United Kingdom
[e] Inserm U781, Hôpital Necker-Enfants Malades, Paris, France
[f] Department of Genetics and Cell Biology, unit Clinical Genomics, Maastricht University, Maastricht, The Netherlands
[g] Department of Neurology, Erasmus MC, Dr. Molewaterplein 10, 3015 GE Rotterdam, The Netherlands
[h] Department of Pediatrics, Catholic University Leuven, 3000 Leuven, Belgium

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ABSTRACT

A multicenter comparison of mitochondrial respiratory chain and complex V enzyme activity tests was performed. The average reproducibility of the enzyme assays is 16% in human muscle samples. In a blinded diagnostic accuracy test in patient fibroblasts and *SURF1* knock-out mouse muscle, each lab made the correct diagnosis except for two complex I results. We recommend that enzyme activities be evaluated based on ratios, e.g. with complex IV or citrate synthase activity. In spite of large variations in observed enzyme activities, we show that inter-laboratory comparison of patient sample test results is possible by using normalization against a control sample.

1. Introduction

The incidence of mitochondrial disorders is estimated to be at least 1 in 5000 (Schaefer et al., 2004; Skladal et al., 2003). The clinical phenotypes associated with mitochondrial disorders are extremely diverse, varying from an early onset multi-systemic disease with rapid deterioration and death at a young age, to a very mild exercise intolerance presenting at a high age (Haas et al., 2007; Zeviani and Di Donato, 2004). This broad clinical spectrum complicates the diagnosis of a mitochondrial disease. Laboratory tests performed on tissue samples, in particular muscle, can provide valuable diagnostic information on the functioning of individual components of the mitochondrial energy generating system. Usually, these tests consist of enzyme activity measurements of the mitochondrial oxidative phosphorylation system. In addition, some diagnostic laboratories perform assays to examine the total mitochondrial energy generating system, including mitochondrial oxygen consumption, substrate oxidation, or ATP production measurements (Janssen et al., 2006; Rustin et al., 1994; Will et al., 2006), although these assays are not possible in frozen biopsy samples. Reaching a diagnosis usually requires that the outcome of these laboratory tests is evaluated in the context of the clinical presentation, metabolic investigations, histological findings, and molecular genetic tests (Taylor et al., 2004). Although a diagnosis is seldom reached on the basis of a single diagnostic test, the biochemical evaluation of a muscle biopsy is generally considered to be the "golden standard" in the diagnosis of a mitochondrial disorder. In addition to muscle, useful diagnostic information can be obtained from other tissues and cell types as well. Some mitochondrial disorders are not expressed in muscle, and require a biopsy of other tissues in order to detect the mitochondrial defect; a liver biopsy in case of an *MPV17* defect is a good example (Spinazzola et al., 2006). In addition, skin fibroblast analysis is often performed.

* Corresponding author at: Nijmegen Centre for Mitochondrial Disorders, 774 Laboratory of Genetic, Endocrine and Metabolic Disease, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands. Tel.: +31 24 3614818; fax: +31 24 3618900.
E-mail address: r.rodenburg@cukz.umcn.nl (R.J.T. Rodenburg).
[1] Equal contribution.

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Fibroblasts have the added value of providing i) important biochemical clues for the identification of a genetic defect, ii) a model system for more in-depth diagnostic analyses, and iii) useful information which may be used to decide whether prenatal diagnostics on the basis of enzyme activity measurements can be performed in families with enzyme deficiencies, where a genetic defect in the mtDNA has been excluded (van den Heuvel et al., 2004).

A direct comparison of results of enzyme activity measurements performed in different diagnostic labs is hampered by the fact that most labs use their own assay conditions and control ranges (Thorburn and Smeitink, 2001). It has been shown before that respiratory chain enzyme activities measured by different labs can show large variations (Gellerich et al., 2004; Medja et al., 2009). Whether the results obtained with these apparently different methods could also lead to different conclusions, has never been tested in patient samples to date, although a recent quality control study using *Caenorhabditis elegans* mitochondrial samples indicates that this might indeed be the case (Chen et al., 2011). The aim of this study was to compare diagnostic methods in the laboratories of 5 diagnostic centers in Europe: Hôpital Necker-Enfants Malades (Paris, France), C. Besta Institute of Neurology (Milan, Italy), Erasmus Medical Center (Rotterdam, The Netherlands), Newcastle Mitochondrial NSCT Diagnostic Laboratory and Wellcome Trust Centre for Mitochondrial Research (Newcastle upon Tyne, UK), and Nijmegen Center for Mitochondrial Disorders (Nijmegen, The Netherlands). The comparison included a detailed examination of sample preparation methods, enzyme activity assays, and data analysis, as well as results from assays performed on a set of patient-derived and control muscle and fibroblast samples. The analysis of the OXPHOS system is invariably included in the examination of patients with suspected mitochondrial disease, while the measurement of enzymes such as pyruvate dehydrogenase is only usually performed in cases with a specific clinical or biochemical indication. For this reason, our study only focused on the biochemical analysis of the OXPHOS system.

2. Materials and methods

2.1. Enzyme activity measurement protocols

Five laboratories participated in this study. A detailed overview of the enzyme activity assays is given in the Supplementary data. The protocols used for the spectrophotometric respiratory chain enzyme activity measurements that were compared in this study are based on the same assay principles, with the exception of the assays for complexes I and II. For these two enzymes, the labs participating in this study used two different types of assays. The NADH-cytochrome c oxidoreductase assay measures complex I–complex III, and under normal conditions complex I is rate-limiting in this assay. Some labs use this assay in parallel with a NADH–CoQ oxidoreductase, in which only complex I is measured. This latter assay is generally regarded to be more difficult to perform, in particular in samples with relatively low mitochondrial mass, such as fibroblasts. Similarly, for complex II both a succinate:cytochrome c oxidoreductase assay (complex II + III) and a succinate:CoQ oxidoreductase assay were used. In this assay, complex II is rate limiting. The combined assays of complex I + III and II + III can also be used to detect primary CoQ deficiencies. Addition of a CoQ analogue to the reaction mixture results in normalization of the activity that is reduced in CoQ deficient samples (Lopez et al., 2006). For all assays that were used in this study, the reaction mixtures show variation in buffer conditions, substrate concentrations, and even temperature, although the assay principles are the same. The enzyme measurements to determine the effect of the buffer in which mitochondrial extracts were resuspended on the outcome of the enzyme activity measurements in fibroblasts were performed following the methods of lab 4 with minor modifications (Rodenburg, 2011).

2.2. Fibroblast sample preparation

Experiments on patient fibroblast sample were performed in accordance with the ethical standards as formulated in the Helsinki Declaration of 1975 (revised 1983). A set of 16 fibroblast cell lines were shipped to each of the participating labs in a blinded manner. This part of the study was coordinated by lab 4. Each center contributed to the study with a number of cell lines. The cell lines are described in Table 2. Each lab cultured the cell lines and subsequently prepared mitochondrial extracts using their routine procedures, with the exception of lab 5 that did not participate in this part of the study because diagnostic fibroblast analysis is not operational in this lab. Furthermore, it should be noted that lab 3 participated although this lab does not offer diagnostic testing of fibroblasts. In each lab, cells were grown at 37 °C in a humidified 5% CO_2 atmosphere, and were trypsinized once or twice a week, and medium was replaced at least once a week. The following culture media were used. Lab 1: RPMI 1640 supplemented with glutamax (446 mg/l), 10% (*V*/*V*) fetal calf serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, 200 μM uridine and 2.5 mM sodium pyruvate. Lab 2: Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 10% (*V*/*V*) fetal calf serum, 1 mM sodium pyruvate, 50 μg/ml uridine, 200 U/ml Penicillin G, 200 mg/ml streptomycin, and 4 mM glutamine. Lab 3: DMEM containing 4.5 g/l glucose, 1 mM sodium pyruvate, 10% (*V*/*V*) FCS, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 0.2% (*w*/*V*) uridine. Lab 4: medium 199 supplemented with 10% (*V*/*V*) FCS, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Preparation of cell extracts from fibroblasts for enzyme activity measurement was performed as follows. Lab 1: a small aliquot of pellet (fewer than 1 million cells; 0.5–0.7 mg protein) was deep-frozen and subsequently thawed using 1 ml of ice-cold solution (medium A) consisting of 0.25 M sucrose, 20 mM Tris (pH 7.2), 40 mM KCl, 2 mM EGTA, and 1 mg/ml bovine serum albumin, 0.01% digitonin (*w*/*V*), and 10% Percoll (*V*/*V*). After 10 min incubation at ice temperature, cells were centrifuged (5 min × 5000 *g*), the supernatant was discarded, and the pellet was washed (5 min × 5000 rpm) with 1 ml of medium A devoid of digitonin and Percoll. The pellet was re-suspended in 30 μl of medium A and used for enzyme assays. Lab 2: a cell pellet of approx. 5 million cells was resuspended in 2 ml Buffer B (250 mM sucrose, 20 mM MOPS KOH pH 7.4). 2 ml of 0.2 mg/ml digitonin in buffer B was added. After incubating on ice for 5 minutes, samples were centrifuged at 5000 ×*g* for 3 min, and pellets were resuspended in 3 ml of 1 mM sodium EDTA in buffer B. After incubating on ice for 5 min, samples were centrifuged at 10,000 ×*g* for 3 min. The pellets were resuspended in 1 ml 10 mM potassium phosphate buffer pH 7.4, and snap frozen in liquid nitrogen and thawed at 37 °C three times before enzyme measurements. Lab 3: to a freshly prepared cell pellet of approx. 5 million cells, 500 μl sucrose–HEPES–EDTA buffer was added. The cell suspension was homogenized by pipetting up-and-down 10 times using a 1 ml Eppendorf pipette. The homogenates were stored deep frozen in small aliquots. This material was used directly in the enzyme assays. Lab 4: a cell pellet of approx. 20 million cells was resuspended in 2.9 ml 10 mM Tris-HCl, pH 7.6 and homogenized using a Potter–Elvehjem tube, after which 600 μl 1.5 M sucrose was added. This mixture was centrifuged for 10 min at 600 *g*. The supernatant was subsequently again centrifuged at 10,000 *g* for 10 min. The mitochondrial pellet was resuspended in 670 μl 10 mM Tris-HCl, pH 7.6. This was used in the enzyme assays.

2.3. Muscle samples

For muscle sample analysis, it was not possible to include the samples from patients with established (molecular genetic) mitochondrial defects because of lack of sample availability. Therefore, it was decided to include 3 control muscle samples to evaluate the reproducibility of the enzyme assays. The control muscle tissue (musculus erector spinae) was collected from patients who underwent a surgery to remove redundant muscle tissue. These patients were not suspected to have a