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Enzyme based diagnosis of type 1 and 2 neuronal ceroid lipofuscinoses

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Abstract: The study aimed to diagnose type 1 and 2 of neuronal ceroid lipofuscinoses (NCLs) using leukocytes and dried blood spot samples. A hundred subjects divided equally to patient and control groups of matching age and sex were included. The patient group was subclassified into two subgroups according to age. Measurement of palmitoyl protein thioesterase1 (PPT1) and tripeptidyl peptidase1 (TPP1) enzymes were conducted in both leukocytes and dried blood spot samples. Chitotriosidase enzyme activity was measured in plasma samples and was found to be normal in 10 cases and increased in 2 cases. Based on PPT1 and TPP1 enzymes activity, 12 cases were diagnosed from the 50 investigated patients and distributed equally between the 2 types. In cases with NCL 1, the Mean ± SD PPT1enzyme activity was decreased to 0.02 ± 0.0074 nmol/h/mg protein in leukocytes samples when compared to 3.4 ± 2.5 nmol/h /mg protein in controls while the activity was decreased to 0.063±0.09 nmol/spot in dried blood spot samples when compared to 0.88±0.32 nmol/spot in controls. In cases, with NCL2 the Mean ± SD TPP1 enzyme activity was decreased to 0.023±0.014 nmol/h/mg protein in leukocytes when compared to 0.92±0.84 nmol/h /mg protein in controls. While the activity was decreased to 0.04±0.035 nmol/spot in dried blood spot samples when compared to 0.394±0.17 nmol/spot in controls. The study concluded that dried blood spot sample method is satisfactory for confirming or excluding suspected patients while leukocyte samples are needed to confirm the positive or unclear results.

Keywords: Neuronal Ceroid Lipofuscinoses; Chitotriosidase; Palmitoyl Protein Thioesterase1; Tripeptidyl Peptidase1; Leukocytes; Dried Blood Spot.

1. INTRODUCTION

The neuronal ceroid lipofuscinoses (NCLs) are a class of common inherited neurodegenerative disorders of childhood[1]. The incidence rate is region dependent and is ranging from 1:25000 to 1:100000[2]. All types of NCLs share two main findings: degeneration of nerve cells and autofluorescent ceroid lipopigments (lipofuscin) assembly in both neural and peripheral tissues[3].

The NCLs are considered as lysosomal storage diorders (LSDs) because of two hallmarks: the lysosomal accumulation of ceroid lipopigments and the lysosomal presence of many NCL proteins [4]. However, the collected material in NCLs is not specific for a disease and subunit c of mitochondrial ATP synthase or sphingolipid activator proteins A and D are the principle storage materials [5].

The NCLs are caused by mutations in thirteen different genes. Mutations in CLNs types 1-3 together account for the broad majority of NCL cases [6].

The CLNs were grouped basically by age at onset: CLN1 as the infantile-onset form, CLN2 as the late infantile-onset form; CLN3 as the juvenile-onset form; and CLN4 as the adult-onset form. Recognition of molecular error changed the classification of CLNs to the numerical form according to the causative genetic defect [7]. Two groups of NCLs can be noticed in this classification: the first group (that comprises 4 NCLs subtypes 1, 2,10and 13) is caused by a deficiency of a protein with a defined enzymatic function, while the second group (that comprises all the other subtypes of NCLs) is caused by deficiencies of proteins with argument or undiscovered role. The diagnosis of the first group of NCLs can be confirmed by measurement of enzymatic activity[8].

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Deficiencies of soluble lysosomal proteins with studied enzymatic functions contribute to four of the distinguished human NCLs. NCL1 (Santavuori-Haltia disease) induced due to palmitoyl-protein thioesterase 1 (PPT1) deficiency; PPT1 is a soluble lysosomal enzyme that separates the fatty acid portion from cysteine remnants of S-palmitolyated proteins[9]**.** NCL2 (Jansky-Bielchowsky disease) is attributed to tripeptidyl peptidase (TPP1) deficiency. TPP1 is a lysosomal serine protease, [10]**.** CLN10 is due to cathepsin D deficiency which is a soluble lysosomal cysteine protease [11]; and CLN13 is caused by mutations in cathepsin F [12].

CLN1 Disease: Infantile neuronal ceroid lipofuscinosis (INCL) has an onset of 6 months up to one year. Cases with this type are presented with deterioration of vision, microcephalic changes, epilepsy, mental and motor retardation generating vegetative phase and death usually occurs in the first ten years of life[13]**.** The CLN1 gene is located on chromosome1p32 and encodes PPT1 protein[14]**.** The PPT1 gene was first discovered in 1995 and about 67 diseases causing mutations have been reported in the CLN1 since [15]**.**

Differential diagnosis includes other progressive neurologic diseases accompanied by onset from childbirth to two years of age these include: hexosaminidase A deficiency, progressive leukodystrophies, Rett syndrome, peroxisomal biogenesis disorders, Neimann-Pick disease A and B, and Leigh syndrome [16].

CLN2 Disease: This type was known as classic late infantile neuronal ceroid lipofuscinosis (LINCL). Deficiency of the TPP1 activity is caused by mutations in the CLN2 gene on chromosome 11p15 [17]. This gene was discovered in 1997 and more than 131 disease-causing mutations have been reported since 2019[19]**.** TPP1 is a lysosomal hydrolase known to detach tripeptides from the N-terminal of small polypeptides [17]. LINCL has two to four years age of onset. Seizures and ataxia are the principle symptoms detected clinically at age of onset, along with progressive cognitive and motor failure. Sight failure shows up by the progression of the disease[18]**.**

Differential diagnosis includes other progressive neurologic diseases with the same age of onset. Suspected diseases are: epileptic encephalopathies, other lysosomal storage disorders, mitochondrial disease and leukodystrophies[16].

Children and young adults who grow up normally at the beginning then later presented with an inexplicable progressive neurological symptoms having mainly dementia, retinopathy, epilepsy, and motor failure are suspected to have NCL disorder. Diagnosis of such disorder (NCL) mainly depends on the age of onset but the absolute diagnosis is molecular testing dependent. NCL types caused by deficiency of enzymes present mainly in lysosomes (iPPT1, TPP1, cathepsin D and cathepsin F) are basically diagnosed by measuring the enzyme activity. In a juvenile-onset disease CLN3 disease are highly suspected, in that case, examination of blood smear looking for lymphocyte vacuole which is a remarkable characteristic for this type. In some cases, electron microscopic can be helpful for examination of blood lymphocytes or tissues to remove doubts about a storage disorder [20]**.**

Stating that NCLs are associated with a massive number of genes mutations, functional enzyme assay for the types caused by the deficiency of these enzymes remain an important tool for the diagnosis of some NCLs types, specifically when variants of unknown significance are detected in cases applying molecular testing[8].

At the time being, all types of NCLs are incurable and supportive treatment is the soul procedure to ameliorate the patient's life quality. Some trials to treat NCLs resulting from soluble lysosomal enzymes deficiency (CLN1, CLN2, CLN10 and CLN13) such as enzyme replacement therapy, gene therapy, and stem cell therapy are still being assessed without sufficient outcome[21]. Enzyme replacement therapy with a recombinant human tripeptidyl peptidase-1 cerliponase alpha (Brineura®) was declared as a treatment for CLN2 patients more than 3 years [22].The medicine is received intracerebroventriculary every other week as an infusions by a reservoir implanted by surgery under the scalp.

This study aimed to biochemically diagnose NCLs types 1 and 2 and compare the results of enzyme activity in leukocytes samples to those in dried blood spot samples in both types.

2. METHODS

Based on the history and clinical examination, 50 Egyptian patients were selected. Patients were referred to the department of Biochemical Genetics at the National Research Centre (NRC) from August 2017 till October 2019. The patients group (50 patients) was subclassified into two subgroups according to the age, the first group comprised patients from age 6 months to 2 years and included 12 patients while the second group included 38 patients of age more than 2 years.

Fifty healthy volunteers of age and sex matching to the study group were considered over the same period of time as control group. All subjects involved in the study gave a written fully informed consent. The study protocol was approved

by the Medical Research Ethics Committee at the NRC (Number: 16430).

After full history taking, pedigree construction and clinical examination, five ml EDTA blood was withdrawn for each patient for determination of enzyme activity either in plasma or leukocytes and a dried blood spot sample was also withdrawn for the same purpose. Each sample was repeated twice for confirmation of the results by a new sample. The samples were subjected to the following:

1. Determination of Chitotriosidase enzyme activity in plasma samples using 4-methylumbelliferyl-β-N-N,N”,-triacetylchitotriose as a fluorogenic substrate and 4-methylumbelliferone as a standard following Hollak et al.,[23].
2. Determination of palmitoyl-protein thioesterase enzyme activity using 4-methylumbelliferyl-6-thiopalmitoyl-β-glucoside as a fluorogenic substrate and 4-methylumbelliferone as a standard, following [24] in leukocyte samples and [25] in dried blood spot samples.
3. Determination of Tripeptidyl peptidase I enzyme activity using Ala-Ala-Phe 7-amido-4-methylcoumarin as a fluorogenic substrate and 4-methylcoumarin as a standard following [26] in leukocyte samples and [25] in dried blood spot samples.

The fluorescence produced from 4-methylumbelliferone or 4-methylcoumarin was read at 365 nm as the excitation wavelength and 450 nm as the emission wavelength using (Hitachi 650-109 fluorescence spectrophotometer; Hitachi Koki Co. Ltd). Results are calculated from the calibration curve (Figures 1 & 2). The protein content of the leukocyte homogenate is measured with the method described by [27]. Results were usually expressed as µmol/l/h for plasma, nmol/h/mg protein for leukocyte homogenates and nmol/spot for DBS.

2.1. Statistical analysis

GraphPad Prism® software for data interpretation was used. Data are introduced as Mean ± SD. Paired Student`s t-test was performed to analyze the statistical significance of the studied cases. A 0.05 level of significance was used, results beneath this level were considered to have statistical significance.

3. Results

Descriptive characteristics of the study groups are presented in table (1). The clinical manifestations of the patient group are presented in table (2).

Chitotriosidase enzyme activity was measured for patient and control groups (table 3). Normal range of enzyme activities in healthy controls was 4–80 µmol/l/h. The level of chitotriosidase enzyme activity was found to be normal in 48 cases and significantly high in 2 cases. Considering the 12 diagnosed cases the level of chitotriosidase enzyme activity was found to be normal in 10 cases with Mean ± SD 46.2 ± 17.85 µmol/l/h and significantly high in 2 cases with Mean ± SD 109 ± 1.42 µmol/l/h. It was noted that the 2 cases with elevated chitotriosidase enzyme activity were diagnosed with NCL 2.

Twelve (24%) cases were diagnosed from total 50 patients according to the results of the enzyme assay and distributed as follow: 6 (12%) with NCL type 1 and 6 (12%) with NCL type 2.

Palmitoyl-protein thioesterase enzyme activity was measured for patient and control groups in both leukocytes and dried blood spot samples, showed in table (4). In healthy controls, PPT1 enzyme activities ranged from 0.52–14.1 nmol/h/mg protein in leukocytes samples. While PPT1 enzyme activities ranged from 0.1–1.5 nmol/spot in dried blood spots. Forty four patients from the patient group showed normal activity. Six cases showed deficient PPT1 enzyme activity.

Tripeptidyl peptidase I (TPP1) enzyme activity was measured for patient and control groups in both leukocytes and dried blood spot samples, showed in table (4). In healthy controls, TPP1 enzyme activities ranged from 0.23–3.9 nmol/h/mg protein in leukocytes samples. While PPT1 enzyme activities ranged from 0.1–0.7 nmol/spot in dried blood spots. Forty four patients from the patient group showed normal activity. Six cases showed deficient TPP1 enzyme activity.

Table (5) showed the characteristics and laboratory findings of the 6 cases with NCL1. The clinical manifestations of the 6 cases with NCL1 showed that all the cases suffered from seizures, five out of 6 cases (83.3%) diagnosed with NCL1 had brain atrophy, regression of acquired milestones and optic problems, while 4 out of 6 cases (66.6%) had ataxia and microcephaly.

Table (5) showed the characteristics and laboratory findings of the 6 cases with NCL 2. The clinical manifestations of the 6 cases with NCL2 showed that all the cases suffered from seizures and regression of acquired milestones, five out of 6 cases (83.3%) diagnosed with NCL2 had brain atrophy, three out of 6 (50%) had ataxia and 2 out of 6 (33.3%) had optic problems, microcephaly was not evident in any of the diagnosed cases.

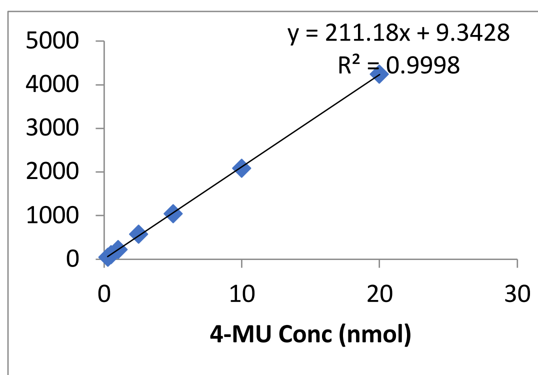

Figure (1): Calibration curve for 4-methylumbelliferone (*4-MU*).

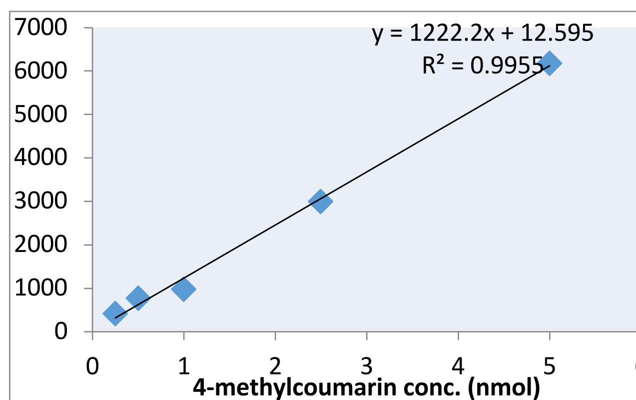

Figure (2): Calibration curve for 4-methylcoumarin.

Table (1): Distribution of the Subjects Characteristics in the patient and control groups.

Subjects Characteristics		Male	Female	Consanguineous parents	No history of any disease in the family	undiagnosed siblings with neurological symptoms	undiagnosed siblings who died early and remained undiagnosed
patient group	n	34	16	38	20	18	12
	(%)	68%	32%	76%	40%	36%	24%
Control group	n	32	18	21	-------	------	------
	(%)	64%	36%	42%			

n = number of cases

Table (2): Distribution of the clinical manifestations in the patient group (50 cases).

Clinical symptom	Seizures	Regression of the acquired milestones	Brain atrophy	Ataxia	Optic involvement	Microcephaly
N	45	42	37	20	20	15
Percentage (%)	90%	84%	74%	40%	40%	30%

n = number of cases

Table (3): Levels of chitotriosidase enzyme activities in the patient group and controls.

Group	Control group n= 50	Patient group	
		High chitotriosidase n=2	Normal chitotriosidase n=48
Chitotrisidase µmol/l/h	34.6 ± 7.9	109 ± 1.4*	40.6 ± 12.9

Data are presented as Mean ± SD, n = number of cases, * p<0.05 against control values.

Table (4): PPT1 and TPP1enzyme activities in healthy controls and patients assayed in leukocytes and dried blood spots samples.

Enzyme Assayed	Type of sample	Controls n=50	Patients with normal activity n=44	Cases with deficient activity n=6
PTT1	Leukocyte	3.37± 2.55 nmol/h/mg protein	3.51±2.58 nmol/h/mg protein	0.0195±0.0073* nmol/h/mg protein
	Dried blood spot	0.88± 0.32 nmol/spot	0.91±0.32 nmol/spot	0.063±0.092* nmol/spot
TPP1	Leukocyte	0.92± 0.84 nmol/h/mg protein	0.95±0.79 nmol/h/mg protein	0.023±0.014* nmol/h/mg protein
	Dried blood spot	0.394± 0.17 nmol/spot	0.41±0.21 nmol/spot	0.04±0.035* nmol/spot

Data are presented as Mean± SD, n = number of cases, * p<0.05 against control values.

Table (5): Characteristics and laboratory findings of cases diagnosed with NCL1 and 2.

NCL type	Number of cases	Sex M:F	Age of onset (y)	Age of diagnosis (y)	Similarly affected siblings	Consanguinity	Chitotriosidase umol/l/h	PPT1 nmol/h/mg protein	PPT1 nmol/spot	TPP1 nmol/h/mg protein	TPP1 nmol/spot
1	**6**	5:1	1.2±0.45	3.75±2.6	83.3%	100%	42±21.35	0.019±0.007	0.063±0.092	2.5±1	0.5±0.14
2	**6**	1:5	3.83±0.75	5.5±1.56	50%	83.3%	71.3±30.3	2.55±1.9	0.87±0.19	0.023±0.014	0.04±0.035

Data are expressed as Mean ± SD, M= male, F=female, y=year.

4. Discussion

Neuronal Ceroid Lipofuscinoses (NCLs) going by the name Batten Disease is a class of progressive, neurodegenerative diseases coexisting with premature death in detected cases. The clinical symptoms may comprise progressive sight failure, a marked decrease in cognitive functions, progressive decline of communicative intelligence, personality and behavioral changes, progressive dropping of motor skills, reduction of muscle mass, deterioration of capability of ambulation, and eventually death[28].

The NCLs are considered as a type of LSDs due to characterization of four human NCLs caused by deficiencies of soluble lysosomal proteins with known enzymatic activity. These four types are CLN1, 2, 10 and 13 are result of deficiency of the soluble lysosomal enzymes palmitoyl-protein thioesterase 1 (PPT1), tripeptidyl peptidase (TPP1), cathepsin D and cathepsin F, respectively[12].

Dried blood spot (DBS) provide the best sample for testing newborn babies. The collection of DBS is minimally invasive. The simplicity of sample collection, transportation, and storage is making DBS a brilliant and cheap choice for many clinical applications [29]. The previous observation encouraged the present study to evaluate the sensitivity for the enzyme activity of both PPT1 and TPP1 in dried blood spot versus that measured in leukocytes.

This study included 50 Egyptian patients and 50 healthy volunteers of matching age and sex. They were referred to the Biochemical Genetics

department at the National Research Centre from 2017 to 2019. The biochemical measurement of the lysosomal enzymes PPT1 and TPP1 was conducted in both leukocytes and dried blood spot samples to detect the biochemical diagnosis of NCL1 and NCL2. These results were further utilized to compare their sensitivity in dried blood spot versus that measured in leukocytes. Measurement of chitotriosidase enzyme activity in plasma samples was also performed. This enzyme is considered as a biomarker for many LSDs.

The gene of NCL2 has more than hundred and thirty different mutations located along the entire TPPI gene [19]and about 67 disease causing mutations have been discovered in the NCL1 gene[15]. This large numbers of mutations makes DNA based diagnosis labor exhaustive and expensive taking into account the time required for either targeted gene or seizure panel sequencing is extremely long compared to an enzyme based assay which offers an easy and fast alternative for either diagnosis or exclusion.

As mentioned by Gavin et al., [28], Batten disease usually presents abnormally making the process of diagnosis complicated and long. Usually, many years are taken from the onset of the first symptoms to the correct diagnosis of the disease. This is consistent with the present study as the Mean ± SD age of onset was 1.2± 0.45 years while the Mean ± SD age of their diagnosis was 3.75± 2.6 years in the 6 NCL1cases while the Mean ± SD age of onset was 3.83± 0.75 years and the Mean ± SD age of diagnosis was 5.5± 1.56 years in the 6 NCL2 cases. It can be noticed that cases spent around 2 years of investigations to be diagnosed. This is also consistent with [30], who stated that most of the LSDs are diversified and have a wide sequence of clinical severity and onset age, making the early detection difficult and leading to prevention of the early diagnosis of these diseases prolonging the time needed for disease detection.

In the present study, the male predominance among the patient group (68%) was evident and is consistent with the work of [31]and this might be due to the oriental culture which gives additional attentiveness to the males giving them particular care. Other Egyptian studies on autosomal recessive diseases like that of[32] showed that M/F was almost 1:1 which matches with the sex distribution in the 12 diagnosed cases in the present study as they showed that M/F was 1:1. This can be an affirmation to increased awareness about the importance of early detection of diseases for both sexes.

Positive consanguinity proved to be 78% within the group under study, while in the dozen cases that were diagnosed the consanguinity percentage was higher at the level of 91.6%. It was found that many previous studies [33-35] claimed that their consanguinity percentage were high above 80%; 75% and 89.9% respectively. The increased percentage of consanguineous marriages between Egyptians could be justified by social, economic and cultural aspects, like preservation of family organization, simplicity of marriage preparations, improved relationship within the same family and better financial support.

In this study 12 cases (24%) were diagnosed out of 50 patients in total; 6 (12%) as NCL1 and 6 (12%) as NCL2. This percentage is considered high when compared with the Brazilian study of [30], who investigated 72797 highly suspected subjects in the period from 1982 to 2015 and 3211 cases (4.4%) were diagnosed with LSD. The higher percentage might be attributed to that, in the present study subjects were referred from a pediatric neurologist specialized in Inborn Errors of Metabolism which made the selection of cases highly suggestive. The other undiagnosed 38 patients could have different types of NCLs that are not due to enzyme deficiency (can be diagnosed on molecular bases only) or other neurodegenerative disorder with similar symptoms such as Tay-Sachs, Sandhoff, Krabbe disease, Metachromatic leukodystrophy, Niemann-Pick disease…… etc. Although, in the entire cases chitotriosidase enzyme activity was measured to exclude some of LSDs. Arylsulfatase A and hexosaminidases enzymes activities were also measured in most of the cases (results not shown).

Chitotriosidase is basically associated with marked lysosomal storage disorders (Gaucher's disease). Gaucher's disease is known to have extremely increased chitotriosidase levels some other LSDs show limitedly elevated levels specifically sphingolipidoses such as Niemann-Pick, GM1 and Krabbe. Chitotriosidase elevated levels was detected in multiplr other lysosomal storage disorders and in some other disorders such as β-thalassemia and sarcoidosis[36]**.**

It was mentioned by[37]**,** that chitotriosidase is known to be an enzyme that is particularly activated in tissue macrophage. This feature of chitotriosidase is making it a possible biomarker for the disease process and prognosis in many lysosomal storage disorders but with different sensitivity. The Mean ± SD chitotriosidase in cases with NCL1 was 42±21.35 umol/l/h, while the Mean ± SD was 71.3±30.27 umol/l/h in cases with NCL2. Cases with NCL2 had significant higher Mean ± SD because two cases had slightly higher level of chitotriosidase than normal this can be justified by the assumption that the process of inflammation and tissue macrophage activation could not have reached to the level that activate the gene to create big quantity of chitotriosidase. The previous

activation was minimal in these 2 cases with NCL2, making chitotrisidase not sensitive and not specific enough in monitoring or diagnosing Neuronal ceroid lipofuscinosis.

As stated by Ostergaard [38], the NCL diseases, generally, usually mentioned within the group of progressive myoclonus epilepsy and progressive neurological deterioration. This agrees with the present study as seizures was the most traditional observed clinical symptom among the study group (90%) and all the twelve diagnosed cases with NCLs (100%) suffered from seizures.

Neuronal ceroid lipofuscinosis constitute a family devastating most common inherited pediatric neurodegenerative disorder globally; one of the clinical hallmarks in this disease is the failure to reach normal milestones and/or developmental demise [39]. The current study focused on regression of the acquired milestones as one of the most distinctive feature of the clinical course of NCLs, it was noted that 84% of the study group presented with regression of the acquired milestones and 11 out of the 12 (91.6%) diagnosed cases with NCLs suffered from this symptom as well.

Dried blood spots are usually used for preliminary tests, whenever feasible, because it aids transportation. Enzymatic activity stays reliable for a prolonged time. Later on, EDTA blood samples are needed for assurance of positive or unclear results[40]. In the present study samples were tested by the two methods in parallel. Each sample was repeated twice for confirmation of the results by a new sample. The 6 diagnosed NCL1 patients showed a Mean ± SD PPT1enzyme activity 0.02 ± 0.0074 nmol/h /mg protein in leukocytes samples while the activity was 0.063 ± 0.09 nmol/spot in dried blood spot samples. The Mean ± SD TPP1 enzyme activity in NCL2 cases was 0.023 ± 0.014 nmol/h /mg protein in leukocytes samples while the activity was 0.04 ± 0.035 nmol/spot in dried blood spot samples (table 4). It was noted that in both methods, there was a significant difference between the patients and the control group. Another observation, was that in both methods the standard deviation for the enzyme activity was higher in dried blood spots method (142.8% of mean in PPT1 and 87.5% of mean in TPP1) compared to the enzyme activity in leukocyte sample (37% of mean in PPT1 and 60% of mean in TPP1). This can be justified by the variability of the results but when taking a closer look to the results, one case diagnosed with NCL1 cases and two cases diagnosed with NCL2 cases showed a higher activity than other cases in dried blood spot samples that remained lower than normal activity. The results of these three cases in leukocyte sample did not differ from other diagnosed cases. However the elevated standard deviation in dried blood spot samples could be attributed to these three cases results.

5. CONCLUSION

The previous observation needs a larger number of patients to confirm that leukocyte sample method is more sensitive than dried blood spot one. It can be concluded that the dried blood spot sample method is reliable as primitive screening test to confirm or rule out cases suffering such disorder considering that this method facilitates transportation, and enzymatic activity stays reliable for a longer period of time. Later, EDTA blood samples are needed to confirm the positive or unclear results in measuring the enzyme activity in leukocyte.

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