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DISTRIBUTION OF NEURAMINIDASE ACTIVITY IN FIBROBLASTS FROM POST-MORTEM SAMPLES

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ABSTRACT

Sialidosis (MIM 256550) is caused by α-N-acetyl neuraminidase (EC 3.2.1.18) deficiency resulting from a mutation in the neuraminidase gene (NEU1) located on chromosome 6p21.33. Currently, samples for the diagnosis of sialidosis were sent out overseas as there is no suitable test available in Malaysia. This study aimed to assess and establish the performance of neuraminidase assay using fibroblasts samples for laboratory diagnosis. Fluorometric measurements of 4-methylumbelliferone-α-D-acetylneuraminic acid (4-MuF-NueAc) were used as an artificial substrate to evaluate the neuraminidase activity. Carbonate buffer pH 10.7 was used as a stopping reagent. The fluorescence intensity of 4-MuF release was measured at a specific wavelength of 366nm excitation and 446 nm emission. Method verification was performed according to the IMR laboratory quality procedure (LQP) guideline. Linearity study showed 4MuF was linear up to 40,000 nmol. Limit of detection and limit of quantitation were 7.998 nmol/hr/mg and 26.66 nmol/hr/mg protein, respectively. Repeatability and reproducibility test results expressed as coefficient of variation (%CV) were 11.38% and 12.52%, respectively. The neuraminidase activity was measured in 8 normal controls and 18 postmortem patients' samples. The median (min to max value) neuraminidase activities in normal and postmortem patients' samples were 38.41 (15.21 to 97.34) and 24.28 (9.49 to 45.77) nmol/h/mg protein, respectively demonstrating a significant difference between both ($p<0.05$). In conclusion, study findings showed that the neuraminidase assay accomplished an appropriate method verification requirement. A new laboratory test for the diagnosis of sialidosis has been effectively established in Malaysia. Nevertheless, more sample size, as well as a separate range between postmortem and living individuals are needed in bringing new insights into this current understanding.

KEYWORDS: Neuraminidase, Sialidosis, 4-methylumbelliferone-α-D-acetylneuraminic acid, Fibroblasts, Postmortem

INTRODUCTION

Sialidosis is a rare autosomal recessive lysosomal storage disease caused by a deficiency of neuraminidase (EC 3.2.1.18) due to mutations in the NEU1 gene located on chromosome 6p21.33. Neuraminidase, also known as sialidase is one of the components in the multi-enzyme lysosomal complex which contains other enzymes such as β-galactosidase and cathepsin A. Approximately, the prevalence of sialidosis is between 1/5,000,000 to 1/1,500,000 live births worldwide (1).

Sialidosis patients were reported to have an impaired degradation of glycoproteins and subsequent accumulation of sialic acid-containing oligosaccharides glycopeptides in the tissues. As a catalyst, neuraminidase removes the terminal sialic acid molecules (N-acetylneuraminic acid or NANA) from glycolipids, glycoproteins and oligosaccharides. Consequently, its mutation disrupts the association of the multi-enzyme complex and subsequent activity or stability which causes the disorder (2).

In humans, four members of neuraminidases have been identified which are NEU1, NEU2, NEU3 and NEU4. All members exhibit different subcellular localisation and substrate preferences (3). Among all, the most studied neuraminidase is NEU1, notably due to its deficiency that is linked to genetic diseases namely sialidosis and galactosialidosis.

NEU1 is a lysosomal neuraminidase that forms a high molecular weight complex with two other lysosomal enzymes; (i) acidic-galactosidase (-GAL) and (ii) serine carboxypeptidase, a protective protein/ cathepsin A (PPCA) (4). For proper folding as well as catalytic activation and stability in lysosomes, NEU1 depends strictly on its association with PPCA (5,6). Thus, NEU1 activity cannot be measured and the enzyme is rapidly degraded in the absence of a functional PPCA. The severe systemic and neurological consequences of NEU1 deficiency in patients with sialidosis represent the importance of NEU1's function for proper lysosomal catabolism in cell maintenance (7,8).

There are two subtypes of sialidosis, (i) Type I, the normomorphic, attenuated form and (ii) Type II, the early onset, dysmorphic form. The clinical symptoms of sialidosis that are present depend on the age of onset and the subtypes of sialidosis. Type I sialidosis is also referred to as cherry red spot and myoclonus syndrome. It is mostly asymptomatic, with signs of myoclonus, seizures, ataxia and visual impairment appearing only in late childhood. As for Type II, the manifestation occurs either within the first year of life with signs including coarse face; enlargement of spleen and liver; dysostosis multiplex; vertebral deformities and severe mental retardation or at birth with a congenital fulminant disease associated with hydrops faetalis; ascites and early death (9,10,11).

At present, there is no effective therapy available for lysosomal diseases. However, innovative approaches have been used to reduce its severity or attenuate the disease progression. The approaches include enzyme replacement therapy and chemical chaperones which provide functional enzymes through endocytosis and stabilising misfolded enzymes therefore, increasing its transportation efficacy to the lysosome, respectively. Other approach, which is still in its infancy was utilising adenoviruses or adeno-associated viruses in gene therapy whereby nucleic acids were transferred into the cells for therapeutic purposes. In lysosomal disease, this normally involves delivering a functional copy of the defective gene using viruses as vectors due to their high chances of allowing gene transfer and expression into any type of cell as well as their potential for infecting nonreplicating cells whilst not integrating with the host genome. However, the most promising approach especially if used at the early stages of the disease, is probably bone marrow transplantation (12,13,14).

Conventionally, sialidosis was diagnosed by measuring neuraminidase activity using enzyme assay in blood leukocytes due to its feasibility during sample collection (15). However, leukocyte was reported to have much lower measurable neuraminidase compared to fibroblasts (16). Furthermore, a low level of neuraminidase along with interference by other enzymes could lead to misdiagnosis (17). Nevertheless, the neuraminidase activity was also demonstrated in a variety of human cells which include erythrocytes, lymphocytes and fibroblasts (18). In order to measure neuraminidase activity, a variety of both natural (sialyllactose, sialylhexasaccharides, and fetuin) and synthetic (3-methoxyphenyl-N-acetylneuraminic acid and 4-methylumbelliferyl-a-D-N-acetyl-neuraminic acid, 4-MuF-NANA) substrates were widely used (12). It has been reported that the accumulation of sialy sialyl glycoconjugates in the lysosomes of various cells derived from patients with NEU1 deficiencies is causing the main clinical manifestations (19).

To date, the diagnosis of sialidosis in Malaysia still relies on metabolite measurement of total and free sialic acid using the thin layer chromatography (TLC) method for screening. However, there are limitations

to this screening method which include the use of hazardous chemicals such as chloroform and hexane; time-consuming;tedious and inconclusive qualitative result. Hence, a quantitative test is needed for disease confirmation.

The unavailability of the enzyme assay testing for neuraminidase in Malaysia requires the physician to send the samples overseas for confirmation of sialidosis. Many suspected patients may be left undiagnosed due to the financial constraint of sending the samples to private laboratories overseas. The physician may also overlook these patients as there is no definitive laboratory testing in Malaysia to diagnose sialidosis. Therefore, there is an urgency to establish this enzyme method to overcome the critically undiagnosed cases in the country. Thus, in this study, we aimed to assess and establish the performance of the neuraminidase assay using fibroblasts samples for laboratory diagnosis.

MATERIALS AND METHODS

Chemicals

Substrate 4-methylumbelliferone-α-D-acetylneuraminic acid (4-MuF-NueAc) was purchased from Toronto Research Chemicals (Toronto, Canada). Sodium acetate buffer, Sodium Carbonate buffer, and Triton X-100 were purchased from Sigma-Aldrich (St Louis, USA).

Fibroblasts sample preparation

The method used was adopted from Kirby (20) with slight modification. Fibroblasts of normal (n=18) and post-mortem (n=8) samples were obtained from archived samples of a previous study (NMRR-17-909-35986) and the forensic departments of various hospitals in Malaysia, respectively. Both cells were cultured under standard conditions in Minimal Essential Medium (MEM) medium with 15% fetal calf serum. The final culture was done in a 175 cm^2 culture flask for 7 days with 5% fetal calf serum for the last 3 days. The medium was then removed, and cells were washed with phosphate-buffered saline (PBS), trypsinised, centrifuged at 100 x g and washed again with PBS. The pellets obtained were kept frozen prior to use at -80ºC. The sonic homogenate was prepared from the fibroblast cell pellet using 500µL deionised water containing 0.1% Triton-X100 using a tip sonicator. The pellet was sonicated on ice for two 5-s bursts at 10% power. This was followed by centrifugation at 10 000 rpm at 4ºC for ten minutes. The supernatant obtained was quantitated for protein quantitation followed by an enzyme assay.

Protein Quantitation

Protein quantification in fibroblasts was determined using the modified Lowry protein assay kit (Thermo Fisher Scientific, Watham, MA, USA). The standard for calibration curve was prepared using Bovine Serum Albumin (BSA), ranging from 1 to 1500 mg/L. A volume of 200 µL of the modified Lowry reagent and 40 µL of either standard or sample was added into the 96 wells clear microplate. The microplate was then incubated for ten minutes at room temperature. After the incubation, 20 µL of Folin-Ciocalteu Phenol reagent was added to each well and incubated for another 30 minutes at room temperature. The protein absorbance was then measured at 750 nm wavelength using Spark 20M multimode microplate reader (Tecan, Mannedorf, Switzerland).

Substrate Preparation

A stock solution of the substrate was prepared according to the method described by O'Brien and Warner (21), 4-MuF-NueAc was prepared by dissolving 0.2 mg in 0.1 mL deionised water. The working substrate mixture was prepared fresh by doubling the volume of substrate stock solution with 0.1 M sodium acetate buffer (pH 4.3) containing 0.1% Triton-X100.

Neuraminidase Assay

The assay was adapted from O'Brien & Warner. The neuraminidase activity was determined with an artificial substrate, 4-MuF-NueAc coupled to sialic acid. Freshly prepared fibroblasts were homogenized with 500 µl deionised water containing 0.1% Triton-X100 prior to usage. 10 µL homogenate sample was added to freshly prepared MU-NeuAc substrate (1:1 dilution with 0.1 M sodium acetate buffer with pH 4.3 and added into 96 wells black microplate. The samples were then incubated for one hour at 37ºC. A volume of 200 µL carbonate buffer was later added into the samples resulting fluorescence product to terminate the reaction and the absorbance was measured by Spark 20M multimode microplate reader (Tecan, Mannedorf, Switzerland) at a specific wavelength of 366 nm excitation and 446 nm emission. The enzyme activities were calculated and compared.

Verification Study

The suitability of this method was assessed by linearity, limit of detection (LOD) and limit of quantitation (LOQ) as well as precision. The verification of the neuraminidase assay was performed according to the Institute for Medical Research Laboratory Quality Procedure (IMR/

LQP) guideline (22).

A series of 4-methylumbelliferone (4-MuF) concentrations were prepared for linearity study. Samples were run in duplicates (Concentration level: 0, 10000, 20000, 30000 and 40000 nmol) prior to data analysis. LOD and LOQ sensitivity were calculated from blank samples which consists of substrate added with the denatured enzyme in replicates (n=12).

Precision study consists of both repeatability and reproducibility assessments conducted by a single operator. Repeatability (n=12) was done in replicates using a single plate at the same time and day (intra-day analysis). Whereas, reproducibility (n=5) was done in triplicate for fiveconsecutive days (inter-day analysis).

We also examined a group of post-mortem fibroblasts samples which was sent to our centre for diagnostic purposes in order to rule out the cause of death of these infants and determine whether an inborn error of metabolism disorder played a role in it (n=18). We selected normal fibroblasts samples (n=8) as the control group.

Statistical Analysis

Data for method verification was analysed using IBM SPSS Statistics version 22 (IBM Corporation) and the results were presented as median (range) with statistical significance at $p<0.05$. Repeatability and reproducibility were calculated on triplicate measurement of their overall mean. LOD was calculated using the following formula: LOD = 3 SD of the blank test (enzyme + substrate)/a where SD is the standard deviation of the response and a is the calibration curve slope. The LOQ was calculated using the following formula: LOQ = 10 SD of the blank test (enzyme + substrate)/a.

For comparisons between groups, Shapiro-Wilk test was applied to determine the distribution of both groups (post-mortem fibroblasts and normal fibroblasts) values in fibroblasts. Data for both groups were not normally distributed and for this reason, we applied a non-parametric test (U-Mann Whitney) at a minimum significance value of $p<0.05$.

RESULTS

Verification Study

Through a 5-point standard curve, linearity was determined and 4MuF, as standard was linear up to 40,000 nmol. The linearity satisfactorily covered the working range of 0 to 2.5 nmol. The linear regression equation curve was y = 125.68x + 190238 with a determination coefficient of 0.993 (Figure 1). Using the spectrofluorometry method, LOD and LOQ values were calculated and estimated at 33 nmol/hr/mg protein and 173 nmol/hr/mg protein, respectively. As for precision, repeatability and reproducibility expressed as coefficient of variation (%CV) in this study were 11.38% and 12.52%, respectively.

Distribution Study

The median age for subjects recruited for the normal fibroblasts sample in this study was two months, ranging from 0 to 33 months old. Whereas, the median age for post-mortem subjects' fibroblast samples was 26 years, ranging from 24 to 32 years old (Figure 2a). Subsequently, gender for normal subjects was divided evenly among males and females with nine persons in each group. Meanwhile, post-mortem subjects consist of six males and two females (Figure 2b).

Distribution of Neuraminidase Level in Study Population

The median (min to max value) neuraminidase activities in normal and postmortem patients' samples were 38.41 (15.21 to 97.34) and 24.28 (9.49 to 45.77) nmol/h/mg protein, respectively demonstrating a significant difference between both ($p<0.05$).

DISCUSSION

Method verification is a process to confirm the suitability for use or confirm the manufacturer's claims of a certain method specification. To ensure quality and certainty, analytical methods provide data and solutions to problems through precision, linearity, LOD and other broad range of analyses.

In verifying the analytical range of assay performance, linearity authenticates the response of the detector to the subjected sample whereby its data should be suited using a linear regression method. The assessment of the regression line quality generally begins with the determination coefficient (R2) which ideally equals one, however, values 0.990 and/or higher are considered acceptable. Factors that may contribute to the absence of linear response include issues with the equipment, solvent, dilution, and complex formation (23,24). Linearity study results showed a linear standard curve with R2 value of slightly more than 0.990, therefore verifying the analytical range.

Sensitivity is defined as the ability to distinguish between small variations of the analyte's concentration.

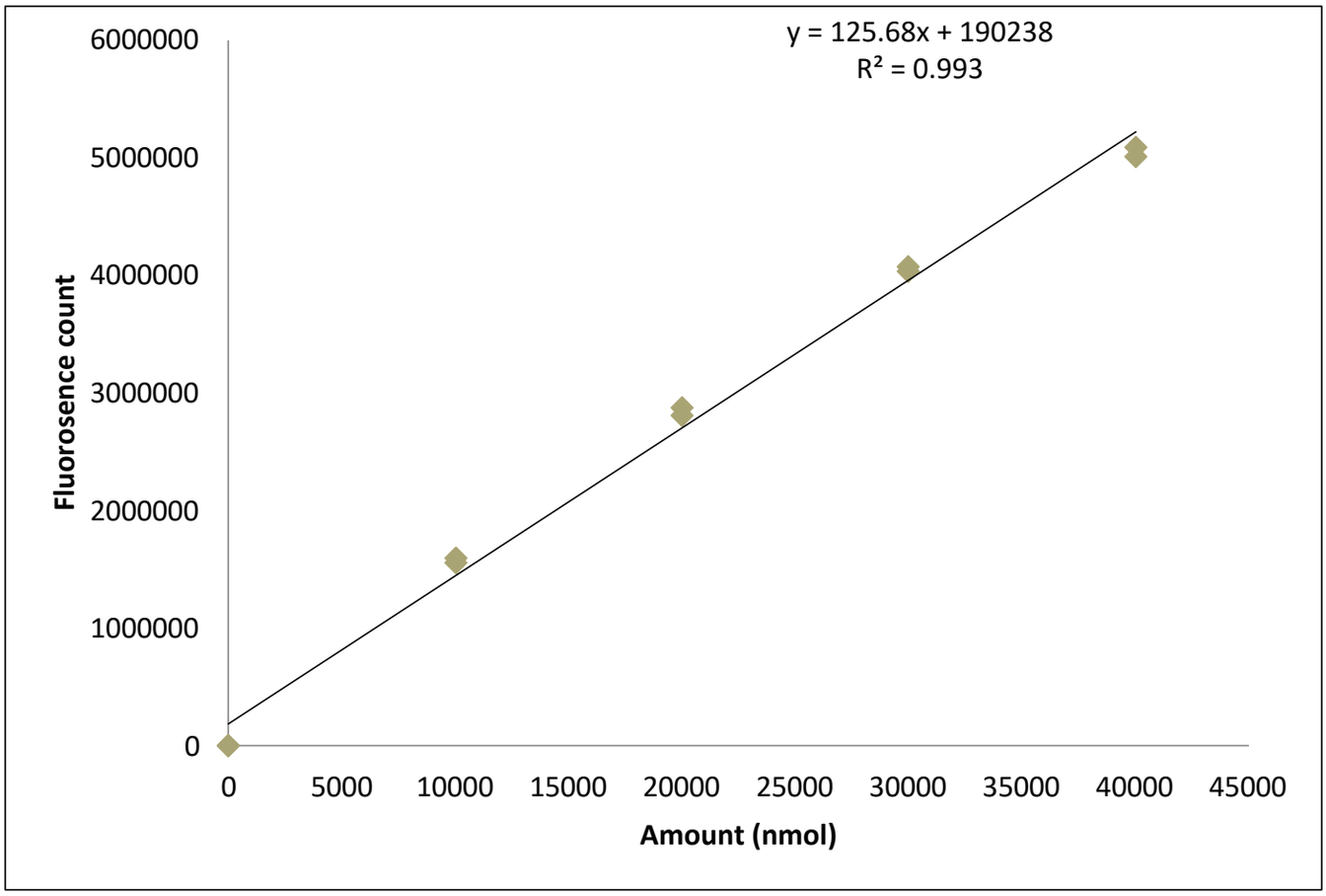

Figure 1: 4MuF linearity standard

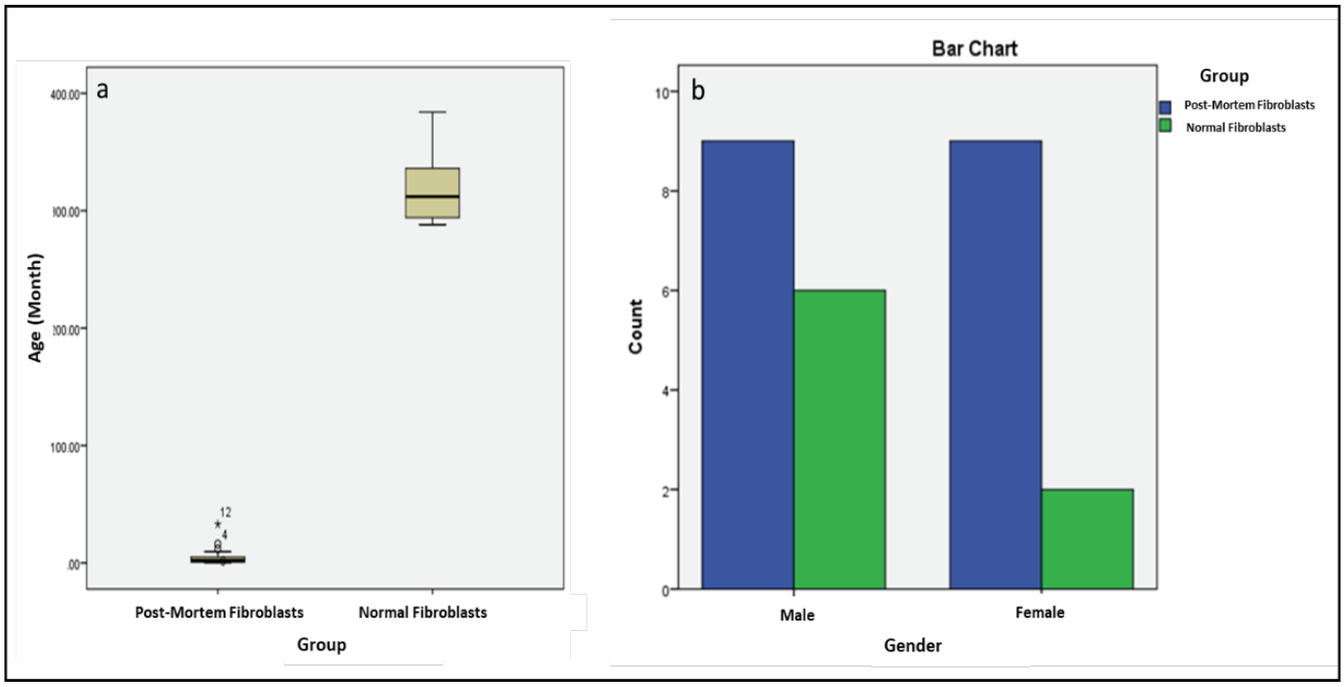

Figure 2: (a) Distribution of age variable between groups (Post-mortem fibroblasts vs Normal fibroblasts) (b) Bar chart between post-mortem fibroblasts and normal fibroblasts for gender variables.

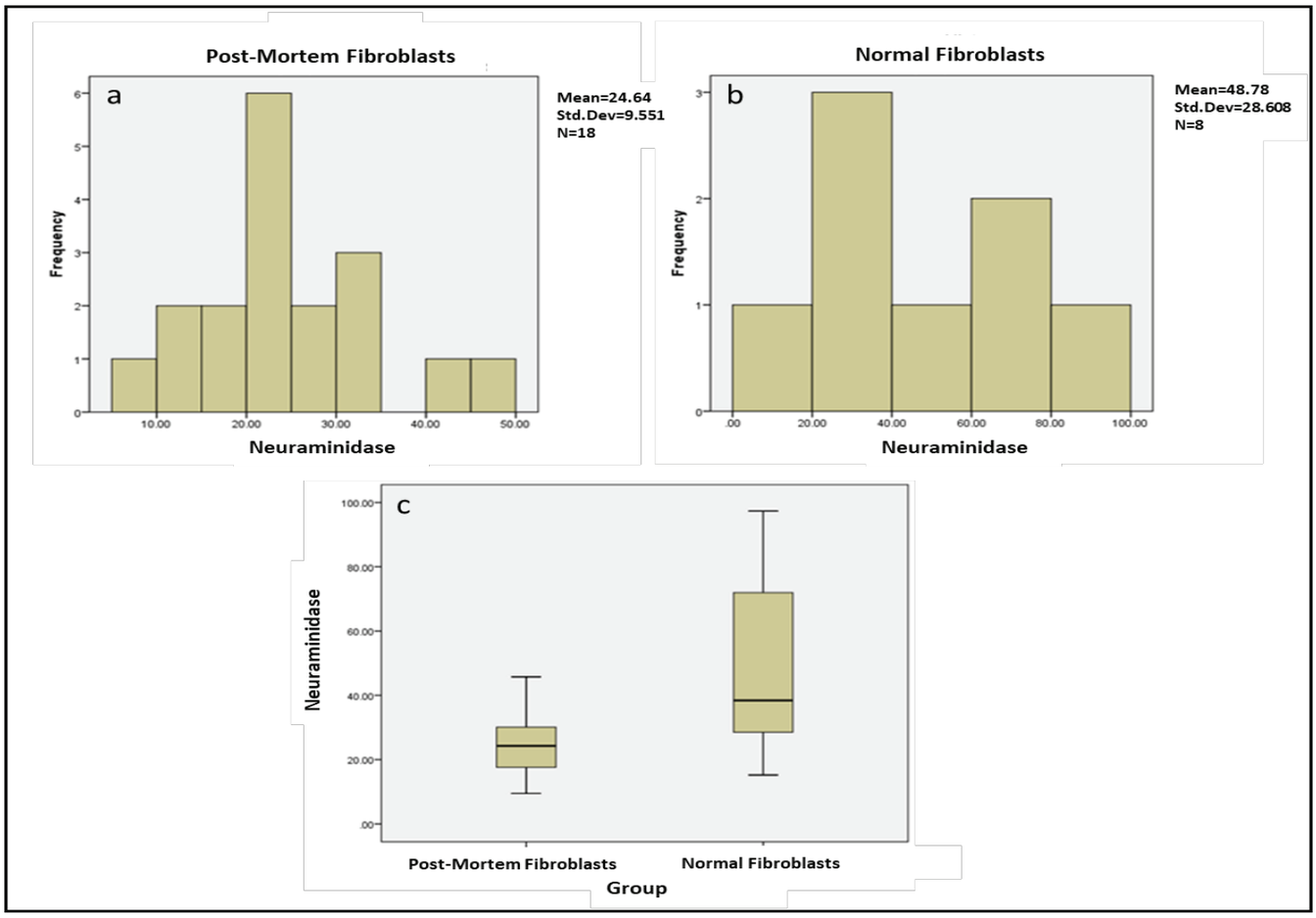

Figure 3: Histogram of neuraminidase activity (a), Post-mortem fibroblasts group (b) and Normal fibroblasts group (c) Comparisons between two groups.

In the establishment of a minimal amount of active ingredient detected (LOD), a significance level of 5% is specified. As for the lowest amount of active ingredient quantified (LOQ), it is determined quantitatively with accuracy and precision under the fixed acceptance criteria (10–20%) (25). According to the European Medicines Agency guideline (26), LOQ could be either equivalent to the LOD or at a much higher concentration. Our results for LOQ were obtained at approximately 5 times the LOD concentration. Hence, the LOD and LOQ are conceded with the respective guidelines.

Variation in repeat measurements made on the same subject under identical conditions is referred to as repeatability while variation in measurements made on a subject under changing conditions is referred to as reproducibility (27). As for precision, which comprises both repeatability and reproducibility assessments, it can be defined as a quantitative expression of random error monitored under specific conditions. As a part of the process for verifying a method, precision confirms its suitability for use (28,29). European Medicines Agency guideline state that imprecision, in general, should not exceed 20% of CV. Based on our results, both repeatability and reproducibility measures were lower than 20% CV; and thus deemed to comply with the guideline.

This study only managed to have a small sample size as we were using available archived normal fibroblasts that were scanty which contributed to the main limitation. This may be the reason for a non-normal distribution of neuraminidase activity among the normal fibroblasts' samples. Alternatively, Kuriyama et al. (15) reported that neuraminidase can also be detected in leucocytes using 2→3 and 2→6 sialylactose as substrates. However, this method is laborious and uses sodium borohydride which is harmful and toxic to users.

Findings from this study also found that the median neuraminidase activity value from the post-mortem fibroblasts samples was lower compared to that of normal living samples.Therefore, in order to further

verify the level of significance, at least 40 samples should be evaluated in future research (22,26).

CONCLUSION

In conclusion, this study showed that the established neuraminidase assay accomplished an appropriate method verification requirement. A new laboratory test for the diagnosis of sialidosis has been effectively established in Malaysia. Nevertheless, larger sample size as well as separating the range between post-mortem and living individuals are required to assist in the exclusion or diagnosis of sialidosis in suspected patients and to bring new insights into this current understanding. Further research should also be done on leucocytes for comparison studies with fibroblasts.

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