

NEW GENERATION TECHNIQUES IN RAPID DIAGNOSIS OF LYSOSOMAL STORAGE DISORDERS

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Article Info

Received 23/10/2015

Revised 16/11/2015

Accepted 09/12/2015

Key words:-

Fluorometry, MS/MS, lysosomal enzymes, diagnostic techniques, inherited metabolic diseases, lysosomal storage disorders

ABSTRACT

Emergence of different therapeutic approaches for lysosomal storage disorders (LSDs) has created interest in early diagnosis and intervention. The diagnosis is mainly done by detecting enzyme deficiency in plasma/ leukocytes/ cultured fibroblasts/ dried blood spots (DBS) by fluorometric/ spectrophotometric methods using artificial/ natural fluorogenic 4 methylumbelliferone/ chromogenic 4 nitrocatechol substrates. The process usually involves complex sampling, testing and validation procedures which causes great difficulties in reaching a definitive diagnosis. Therefore in recent years, efforts are being made to transform the conventional fluorometric methodology of low precision into microchip and immune capture assays using single/ multiplex platforms- digital micro fluid (DMF) and Luminex respectively for rapid screening of LSDs. Implementation of automated systems (MS/MS & LC-MS/MS) are enabling the simultaneous screening of number of LSDs in a single analysis at early course of disease. But every technique has its own advantages and limitations. In present study, we have shared 10 yrs of our experience (2004-2014) in the diagnosis of 1120 cases (24.6%) for 25 different LSDs out of 4542 suspected individuals whose samples received in our genetic centre using conventional fluorometric assays (single analyte analysis technique). Diagnostic efficiency of these assays is compared with the data reported by other researchers from various countries using latest high through-put technologies. Though, fluorometric enzyme assays have been referred as the gold standards for timely diagnosis followed by molecular confirmation, MS/MS based assays seem to have great clinical prospects in future for the diagnosis of IMD due to multiplexity, high precision and specificity. Our experience states that with the advancement of the technologies, there is a need of a defined quality assurance program for the laboratories/ health professionals in order to provide such services. Accurate diagnosis at early stages of life will have a great impact on genetic counselling for further management of the disease.

INTRODUCTION

Lysosomal storage disorders (LSDs) are a group of rare genetic disorders that result from defects in lysosomal function [1]. These are routinely diagnosed by measuring lysosomal enzymes in plasma, leukocytes, skin fibroblasts and dried blood spots [2, 3].

Recently, prenatal diagnoses of lysosomal storage disorders based on conventional fluorometric methods using uncultured chorionic villi samples have been published by Verma et al. [4]. The emergence of effective therapeutic strategies has initiated interest in early and reliable diagnosis by new techniques for better intervention. Present study is intended to understand the merits and demerits of emerging techniques [LC-MS/MS, Digital micro fluids (DMF), Immune quantification, Fluorometry, Next Generation dried plasma spots technology (NGDPST)] for screening / diagnosis of

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lysosomal storage diseases to decide which technique should be used. The age old conventional fluorometry enzyme assays (currently in use at our genetic centre) have been used as a comparator to the recently introduced MS/MS and other techniques. The chemistry applied to diagnose the disease, technical complexity with specificity and sensitivity of the techniques, turnaround time for the reporting and requirement of the infrastructure and clinical utility have been compared to make available the information of all techniques in one study.

MATERIAL AND METHODS

In the present study, 10 yrs laboratory data generated by performing fluorometry enzyme assays for the diagnosis of LSDs in suspected cases in our genetic centre were compared with the data analyzed by other scientists/ researchers from various countries using different analyzers and techniques to screen/ diagnose LSDs [5-14, Figure 1]. The countries included India, Australia, Austria, Taiwan, Washington, Chicago, Minnesota and New England to obtain relevant data. Fluorometric enzyme assays for diagnosis of various LSDs were performed in whole blood leukocytes/ plasma/ dried blood spots/ cultured fibroblasts samples using 4-methylumbelliferyl (fluorogenic) / 4-nitrocatechol (non-fluorogenic) substrates with quality assurance by Verma et al. [14]. After incubating and stopping the reactions, fluorescence readings (excitation 365 nm, emission 455 nm) of 4-methylumbelliferyl were measured using a Victor 2D multi-label counter (Perkin Elmer) and corrected for blanks. A standard curve of 4-methylumbelliferyl (concentration vs. fluorescence) was used to extrapolate the fluorescence count to moles of enzymatic product. Similarly, the standard curve of 4-nitrocatechol (concentration vs. absorbance) was used to extrapolate the absorbance value (non- fluorogenic assay) to moles of enzymatic product. Enzyme Activity was reported in nmol/hr/mg or nmol/17hrs/mg or nmol/4hrs/mg of protein as per the relevant protocols. For each enzyme, one negative (normal subject) and one positive (affected case) sample was run.

Informed consent: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Declaration of Helsinki (2013) of the World Medical Association. Informed consent was obtained from all patients/ normal subjects for being included in the study.

RESULTS AND DISCUSSION

In the present study, diagnostic efficiency of modified fluorometric manual enzyme assays has been compared with results obtained using other major technology platforms e.g. MS/MS or LC-MS/MS multiplex enzyme assays, DMF (Advanced Liquid Logic, Inc., Morrisville, North Carolina) assays and Luminex

(immune-quantification assays) for estimation of lysosomal enzymes to know which technique should be used. All new techniques are sensitive with better prospects in future for newborn screening however, defined criteria for quality control is required at the pre analytical, analytical and post analytical stages [15].

Enzyme replacement and bone marrow transplant therapies are now available for several LSDs and other therapies are under development giving new hope for affected individuals. Therefore, emergence of new technologies for simultaneous screening of these disorders in DBS in multiplexed manner has paved the path for including them in newborn screening programs. DMF cartridge configured with an electronic circuit board for automated droplet handling and a built-in fluorometer. The calibration, incubation and fluorescence measurement are programmed in the software and performed on the cartridge. Currently, 44 DBS samples per cartridge for 5 LSDs (Pompe, Fabry, Hunter, Gaucher and Hurler) can be run in 3 hrs with false positive rate of 0.33 and detection rate 1:890 [16]. Luminex technology is not utilized much in laboratories except for pilot study to compare different methodologies due to the limited availability of antibodies [9]. Specific substrates and antibodies capture assays made accurate analysis of 12 lysosomal disorders. The false positive rate on using LC-MS/MS technology is much lower than the other technologies (Table 1). Tandem mass spectrometry (TMS) has a great advantage in using substrate that are closer to natural substrate and can detect multiple enzyme products simultaneously because each product has a different m/z. The most notable methodological development is that optimized reagent cocktails containing premixed substrates and internal standards are commercially available [17].

In figure 1, chemistry used in various techniques (fluorometry, MS/MS or LC-MS/MS, Luminex and DMF) to perform the enzyme assays for the diagnosis and screening of LSDs has been demonstrated and the most recent technology NGDPST used to collect the plasma sample in very less amount on the easily transportable filter paper after drying for the diagnosis of number of diseases from one spot, has been depicted. Using fluorometer, most of the enzymes can be assayed in blood (leukocytes or serum/plasma) and culture fibroblasts using commercially available synthetic 4 methyl umbelliferone (4 MU) substrates. Though the concept of using dried blood spot (DBS) extracts for lysosomal enzyme testing, as pioneered by Chamoles and colleagues in early 2000s, opened up the way for NBS of LSDs [18], multiplex capacity is limited as the enzyme reactions for all produce same end product 4 MU. In addition, artificial substrates might not necessarily represent their activities *in vivo*; both false negative and false positive may occur [19]. In case of pseudo deficiency, molecular study is important.

The performance of various techniques using complex analyzers LC-MS/MS, Luminex, Lab on-chip (DMF) and fluorometer have been evaluated and



demonstrated in Table 2. Most of the studies reported data for Gaucher, Pompe, MPS 1 and Fabry diseases. In last ten years, we have biochemically diagnosed 1120 LSDs patients out of 4542 suspected cases attended at our genetic centre and confirmed by molecular studies where feasible. We also found Gaucher, Pompe, Krabbe, Tay Sach, Niemann Pick and Mucopolysaccharidoses as the most common disorders in our study. Among various techniques included in this article, no assay seems sufficiently specific at present for diagnoses of LSDs.

Combining assays (screening + biochemical enzyme assays+ mutation study) help to reduce the false positive rate. We have recently published data on prenatal diagnosis of LSDs using conventional fluorometric assays [4]. Based on molecular confirmation of biochemical diagnoses / telephonically/ post natal analysis, 99.5% accuracy was reported with false positive rate 0.5%. With MS/MS, different cutoffs need to be established for different subpopulation of newborns, if used to diagnose at early stages to minimize false negatives without an excessive number of false positives. False positives cause parental anxiety and are expensive in terms of professional time and efforts to obtain repeat specimens for retesting and follow up [19-21].

Chamoles et al. (2004) have reported that MS/MS based DBS assay does not discriminate between infantile and late onset of Pompe disease using fluorometric methods [22] while Chien et al. (2008) found low recall rate in newborn screening for Pompe disease by MS/MS because of its high discriminating power between normal and affected individuals [23]. DMF provides rapid, multiplexed enzymatic analysis of acid α - glucosidase and acid α - galactosidase to screen Pompe and Fabry disorders. It is a low cost alternative to enzymatic methods currently

used for recognition of LSDs with false positive rate 0.33%. Since most of the currently introduced technologies are filter paper based, Shimadzu scientific Instruments in collaboration with Novilytic Labs has offered a powerful new technology for rapid generation of plasma from whole blood for MS based analysis without compromising the assay reproducibility and selectivity [24]. It eliminates the need of time consuming techniques such as solid phase extraction, centrifugation and evaporation. This technology will be highly useful in future as micro quantity can be used.

It has been experienced that with the advancement of the technologies, there is a need of a defined quality assurance program for the laboratories performing enzyme assays to diagnose the complex disorders like LSDs. External quality assurance program (EQAS) for lysosomal enzymes are available with ERNDIM, The Netherlands. We have shown evaluation of our lab EQAS performance (2015) in Table 3.

We performed 60 enzyme activities for the diagnoses of 6 samples and reported 100% correct analysis with no false positive or false negative results. Compliance with the defined internal QC criteria is must for providing reliable results. The major concern regarding accurate and timely diagnosis include pre-analytical factors (sample collection, adequacy and transportation), analytical methods (method standardization, validation, establishment of own cut offs, updated reference intervals for normal subjects and patients, interpretation of results in context with method limitations and clinical details) and finally post analytical methods (reporting the results and informing to patient or relative), all influence the performance of the lab results and have a great impact on genetic counselling for further management of the disease.

Table 1. Technical evaluation and performance of various techniques for the enzyme assays

Which one should be used?

Techniques/ Platforms	LC-MS/MS (Gelb et al. 2006, Scott et al. 2012 , Mechtler et al. 2012)	Luminex (Fuller et al. 2011)	“Lab on a chip” Burton et al., 2012)	Fluorometry (Chamoles et al. 2002, Hwu et al. 2009, Chien et al. 2009, Lin et al. 2009), SGRH, 2004- 2014- (Verma et al. 2015)
Method	Multiplex Enzyme assay	Immune Quantification	Digital Micro- fluidics (DMF)	Enzyme assay (single)
Purpose	Screen	Screen	Screen	Gold standard for definitive diagnosis
Multiplex	yes	yes	yes	no
Sample	DBS	DBS	DBS	DBS, fibroblasts, leukocytes, Chorionic villi/Amniotic fluid
Complexity	high	Low	Low	Low
Cost Effective	Yes : Screening No: Individual Assays	No	Yes	Yes
Infra-structure	Yes	No	No	No
Economical	No	Yes	Yes	Yes



Standardization/ Validation	Standardized for a limited disorders	Standardized for a limited disorders	Standardized for a limited disorders	SGRH: Standardized and well established reference ranges for 25 lysosomal enzymes
Performance	Satisfactory	?	?	SGRH: Satisfactory as certified by "ERNDIM"
Study	Austria (n=35000) Gaucher, Fabry, Pompe, Niemann Pick, MPS I and MPS II (Mechtler et. al (2012) [0.003-0.05% +ve] Washington (n=110000) Pompe, Fabry, MPS1 (Scott et al. 2012) [0.01% +ve]	(n=1415) Fabry, Pompe, MPS 1, 2, 3a, 3b, 6, MLD, NP, MSD, Gaucher, MLII/III, 1 false positive for MPS II (Fuller et. al. 2011)	(n=8012) Pompe, Fabry, Gaucher (Burton et al. 2012)	Fabry (0.87%) / Pompe (0.83%) screen (Hwu et al. , Chien et al. , Lin et al 2009, Taiwan)
				SGRH (2004- 2014): 1120 cases have been diagnosed out of 4542 for 25 LSDs. Prenatal diagnosis (PND) : 331 with 99.5% accuracy
% FPR	0.07 (Austria), 0.023 (Washington), 0.9 (Minnesota)	0.1	0.33	SGRH: 2012-2015: Cultured fibroblast: 4.3% diagnostic error rate (ERNDIM) , PND: FPR: 0.5%, DBS (ERNDIM, Pilot study) : 91.7% accuracy Other countries: 0.86,
% PPV	40 (Austria), 33 (Washington), 21 (Minnesota)	NA	26	3 (other countries)
D. Rate	1:2315 (Austria), 1:7800 (Washington), 1:431 (Minnesota)	NA	1:890	1: 3426 (Other countries)
Unnecessary evaluation/ month	6/ 35000 (Austria), 2/110000 (Washington), 75/100000 (Minnesota)	NA	27	71 (Other countries)
Future	Great potential for screening/ diagnosis with high precision and specificity	Due to the limited availability of antibodies, not very practical	Limited disorders, precision required	Highly approachable, economical, good precision with limitation of single analysis.

Table 2. ERNDIM, The Netherlands, EQAS results for the year 2015: lysosomal enzymes in cultured fibroblasts using fluorometry manual enzyme assays.

Cycle	Year 2015	Disease	Total labs	Apr	May	Jun	July	Oct.	Nov.
S.No	Parameter			Z score	Z score	Z score	Z score	Z score	Z score
1	Protein		66-71	0.22	0.55	0.36	0.87	0.29	-0.46
2	α - galactosidase	Fabry	58-63	0.36	-0.097	0.14	0.003	0.47	-0.32
3	Gal 6 sulfatase	MPS IVa	37-41	0.39	0.033	-0.42	-1.12	-0.06	-0.052
4	β -Galactosidase	GM1	61-66	-1.12	-0.61	-1.1	-0.92	1.04	-1.3
5	α - Glucosidase	Pompe	44-48	-0.51	-0.33	-0.1	-0.08	0.15	0.01
6	β -Glucosidase	Gaucher	57-62	-0.84	-0.79	-1.2	-0.93	-0.69	-0.95
7	β - Hexosaminidase A	Tay Sach	53-57	-0.43	-0.39	-0.3	-0.38	0.22	-0.43
8	β - Hexosaminidase A+B	Sandoff	55-59	-0.86	-0.16	1.2	0.86	1.26	1.9
9	α - Iduronidase	Hurler (MPS1)	48-54	-0.68	-0.56	-0.68	-0.39	-0.53	-0.53
10	Galactocerebrosidase	Krabbe	42-45	1.4	0.02	1.15	-0.81	2.6	0.83
11	Sphingomyelinase	Niemann	41-44	0.28	1	0.26	0.46	1.3	-0.01



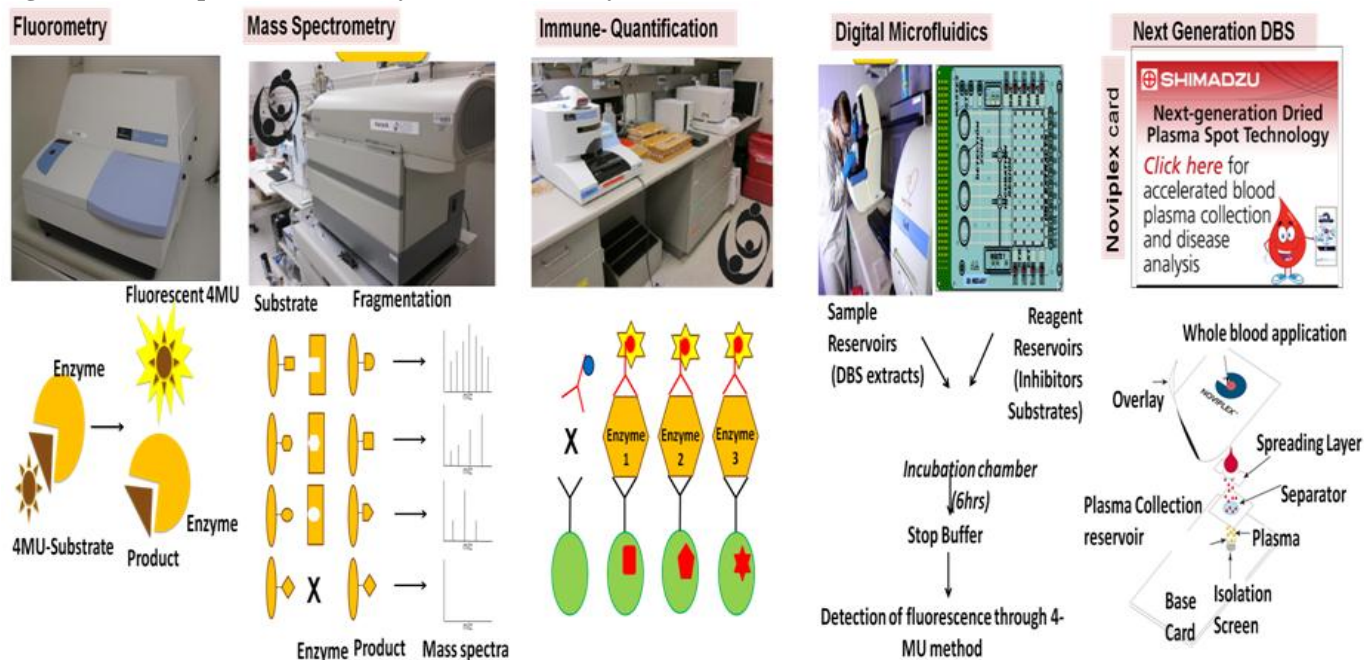
		Pick							
	*Diagnosis			Pompe	Gaucher	Tay Sach	Morquio IVa	Hurler	Pompe

Foot note:

$$Z \text{ score: } \frac{\text{Mean of laboratory value} - \text{Mean of all participating laboratory results}}{\text{Standard deviation of all lab results}}$$

*There are no false positive and false negative results.

Figure 1: Techniques and chemistry used for the assays



CONCLUSION

Mass spectrometry multi analyte technology is emerging as an effective tool for diagnosis of multiple disorders in a single analysis. With the advent of the improved reagents and specific substrates, LC-MS/MS has a great potential for effective screening and fast diagnosis of LSDs with high specificity and sensitivity. However, in most of the developing countries including India, diagnoses are mainly made on a case to case basis by relying on clinical phenotypes and laboratory test results. As a consequence, fluorometric enzyme assays based diagnosis remains the most preferable approach for LSDs. These fluorometry enzyme assays are in expensive to set up, simple to perform and produce reliable results. They are the only available methods used to perform diagnosis in various biological samples in addition to DBS. Prenatal diagnosis using uncultured chorionic villi and cultured fibroblasts is only possible by this bench based fluorometric technique. If work load is high and funds are available, we recommend LC-MS/MS technology with multi- analytes advantage for diagnosis of rare genetic disorders.

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ACKNOWLEDGEMENT

We sincerely acknowledge the work of Gelb, Scott, Mechtler, Fuller, Burton, Hwu, Chien, Lin and their colleagues from Washington (USA), Austria, Australia, Chicago, Taiwan respectively, whose publications were of immense help in the preparation of this manuscript. We thank all patients and normal subjects whose blood was used as a control/ reference material to generate the data for this study. We extend our thanks to Genzyme (India) who has chosen our centre as a referral laboratory for lysosomal enzymes testing. We express our gratitude to doctors from India and neighbouring countries for referring patients to our genetic centre.

Conflict of Interest: The authors declare that they have no conflict of interest. All authors have given final approval for the publication of this article.

Funding: The present work is not supported by any funding agency.



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