Malaysian J Pathol 2010; 32(1) : 35 – 42

ORIGINAL ARTICLE

Separation of sulfated urinary glycosaminoglycans by high-resolution electrophoresis for isotyping of mucopolysaccharidoses in Malaysia

*NOR AZIMAH Azize *BSc*, **ZABEDAH Md Yunus *MPath*, *NORSIAH Md Desa *MSc*, ***NGU Lock Hock *MRCP* and **SUHAILA Abd Rahman *Dip.*

**Molecular Diagnostics and Protein Unit, **Biochemistry Unit, Specialised Diagnostics Centre, Institute for Medical Research, Kuala Lumpur and ***Genetics Department, Kuala Lumpur Hospital.*

Abstract

Mucopolysaccharidoses (MPS) are a group of inherited disorders caused by the deficiency of specific lysosomal enzymes involved in glycosaminoglycans (GAGs) degradation. Currently, there are 11 enzyme deficiencies resulting in seven distinct MPS clinical syndromes and their subtypes. Different MPS syndromes cannot be clearly distinguished clinically due to overlapping signs and symptoms. Measurement of GAGs content in urine and separation of GAGs using high-resolution electrophoresis (HRE) are very useful initial screening tests for isotyping of MPS before specific enzyme diagnostics. In this study, we measured total urinary GAGs by a method using dimethylmethylene blue (DMB), and followed by isolation and separation of GAGs using high resolution electrophoresis (HRE) technique. Of 760 urine samples analyzed, 40 have abnormal GAGs HRE patterns. Thirty-five of these 40 cases have elevated urinary GAGs levels as well. These abnormal HRE patterns could be classified into 4 patterns: Pattern A (elevated DS and HS; suggestive of MPS I, II or VII; 16 cases), Pattern B (elevated HS and CS; suggestive of MPS III; 17 cases), and Pattern C (elevated KS and CS; suggestive of MPS IV, 5 cases), and Pattern D (elevated DS; suggestive of MPS VI; 2 cases). Based on the GAGs HRE pattern and a few discriminating clinical signs, we performed selective enzymatic investigation in 16 cases. In all except one case with MPS VII, the enzymatic diagnosis correlated well with the provisional MPS type as suggested by the abnormal HRE pattern. Our results showed that GAGs HRE is a useful, inexpensive and practical first-line screening test when MPS is suspected clinically, and it provides an important guide to further enzymatic studies on a selective basis.

Keywords: Glycosaminoglycans (GAGs), mucopolysaccharidoses (MPS), keratan sulfate (KS), chondroitin sulfate (CS), heparan sulfate (HS) and dermatan sulfate (DS).

INTRODUCTION

Glycoaminoglycans (GAGs) are composed of long sugar chains containing repeating units of highly sulfated, alternating hexuronic acid and hexosamine residues. Depending on the composition of the repeating units, four different types of GAGs are known - keratan sulfate (KS), heparan sulfate (HS), dermatan sulfate (DS) and chondroitin sulfate (CS). GAGs are essential components of bones, cartilage, skin, tendons, cornea, cardiac valves and synovial fluid.[1]

Mucopolysaccharidoses (MPS) are a group of inherited disorders caused by deficiency of specific lysosomal enzymes involved in GAGs degradation. These enzyme deficiencies lead to the accumulation of GAGs in the lysosomes of cells, resulting in cell, tissue and organ dysfunction. Currently, there are 11 enzyme deficiencies resulting in seven distinct MPS clinical syndromes and their subtypes.[1] The enzymes involved are α-L-iduronidase (MPS I Hurler, Scheie, Hurler/Scheie), iduronate sulfatase (MPS II), heparan *N*-sulfatase (MPS IIIA), α-*N*-acetylglucosaminidase (MPS IIIB), acetyl-CoA-glucominidase acetyltransferase

Address for correspondence and reprint requests: Nor Azimah Abdul Azize, Molecular Diagnostics and Protein Unit, Specialised Diagnostics Centre, Institute for Medical Research, 50588 Jalan Pahang, Kuala Lumpur, Malaysia. Tel: +60326162793; Fax: +60326938210. Email: azimahazize@imr.gov.my

(MPS IIIC), N-acetylglucosamine 6-sulfatase (MPS IIID), galactose 6-sulfatase (MPS IVA), α-galatosidase (MPS IVB), arylsulfatase B (MPS VI), α-glucuronidase (MPS VII) and hyaluronidase (MPS IX).[1,2] Different MPS syndromes share many clinical similarities. Most of them are characterized by multisystem involvement including skeletal abnormalities, stiff joints, macrocephaly, coarse facies, psychomotor delay, neuro-regression, corneal clouding, hearing loss, hepatosplenomegaly and cardiac dysfunction.[1,2,3]

Definitive diagnosis of specific type of MPS syndrome is important because specific treatments such as haematopoietic cell transplantation or enzyme replacement therapy are available for some.[1,3] Although it is possible to differentiate each type clinically to a certain extent, a definitive diagnosis of specific type of MPS can only be achieved by enzyme analysis. It is not feasible to examine all the 11 enzymes for every suspected patient. A simple screening test, along with clinical features, is needed to narrow down the list of potential patients and enzymes to be assayed. It is known that all MPS lead to increased urinary GAGs excretion. It is also known that different enzymes deficiencies lead to different urinary GAGs excretion patterns. Increased amount of DS and HS are excreted in MPS I and II; HS in all MPS III subtypes; KS in MPS IV; DS in MPS VI; CS, DS and HS in MPS VII.[2] Therefore, a comprehensive quantitative and qualitative analysis of urinary GAGs is the first step to diagnose MPS in suspected patients before further confirmation by specific enzyme assays.[4]

Currently in Malaysia, a significant number of MPS patients are diagnosed based solely on clinical signs and symptoms, and skeletal radiological findings because the diagnostic tests need to be outsourced from overseas laboratory. Our aim is to establish a local diagnostic facility for patients with suspected MPS. In this study, we have established a quantitative analytical method to measure the total urinary GAGs and subsequently characterized the isotypes of MPS by separating the urinary GAGS using high resolution electrophoresis (HRE).

MATERIALS AND METHODS

Sample

From 2005 to 2008, 760 urine samples were obtained from patients who exhibited signs and symptoms of MPS from Institute Paediatrics, Kuala Lumpur Hospital and other hospitals in Malaysia. They were paediatric patients of age between 11 days to 15 years. The urine samples were collected in sterile bottles without any preservatives and stored frozen at -80°C until analysis.

Equipment

Discontinuous Electrophoresis System, 15°C cooling water system and power pack.

Materials

Sample applicator; cellulose acetate plate (Titan III 94 x 76 mm); Whatman filter paper; buffers: cetylpyridinium chloride (CPC)/citrate, 0.1 M Barium Acetate, 1% v/v citric acid, 2 M lithium chloride, ethanol, phenol red and alcian blue 8GX; GAGs standards: CS (99% bovine cartilage) and HS (sodium salt from bovine kidney).

Method

Quantitation of urinary GAGs

Urine creatinine concentration was measured using a chemistry analyzer. Urinary total GAGs content was measured using dimethylmethylene blue dye (DMB). GAGs in urine were calculated as per mmol/L creatinine. Volume of urine (ml) used to precipitate at least 600 μg GAGs was calculated using the formula; 45/ [GAGs/urine (mg/L)] x 2.

Isolation of GAGs

Pre-treatment of urine was performed essentially by the method described by Hopwood and Harrison (1982)[5] with some modifications. The calculated amount of urine used was centrifuged at 3000 rpm for 15 min at 25°C to remove debris. Equal volume of CPC/citrate buffer was added to urine samples, mixed and incubated in the water bath at 37°C for 30 minutes. The resulting CPC-GAGs complex was separated by centrifuging at 4500 rpm for 10 minutes at 25°C. The supernatant was decanted and the centrifuge tube drained inverted for 5 minutes. The pellet was dissolved in 150 μl 2M lithium chloride and mixed with 800μl ethanol followed by vortexing it at full speed for 5 seconds or until pellet is well mixed. The mixture was then transferred into 1.5 ml micro-centrifuge tube, let stand for 15 minutes at room temperature, then centrifuged at 3500 rpm for 15 minutes at 25°C. The supernatant was then decanted and the pellet containing GAGs was dried by inverting the microcentrifuge tube on a tissue paper. 10μl of 0.5 g/L phenol red was then added

onto the pellet, vortexed vigorously to dislodge the pellet and dissolve the GAGs. The dissolved GAGs sample was stored frozen before HRE was performed.

High resolution electrophoresis (HRE)

HRE was performed essentially by the method described by Hopwood & Harrison (1981)[5] and Cappelletti *et al* (1979)[6] with minor modifications. The high resolution is achieved by making use of the different solubility of each GAGs in different concentration of ethanolic barium acetate buffer. The electrophoresis system was pre-cooled to 15°C. Cellulose acetate plate was initially immersed in 0.1 M barium acetate aqueous, pH 5.0 and 3 mm Whatman paper was applied to form a wicks and the system was pre-electrophoresed for 30 minutes in 1 L 1 M barium acetate buffer, pH5.0 at 180 V constant voltage. Constant voltage of 180 V was used throughout the electrophoresis. By using a sample applicator, 0.15 mg of each standard and 2 μl (equivalent to 120 mg) of GAGs were loaded onto the cathode side of cellulose acetate plate which had been pre-soaked in 0.1 M aqueous barium acetate, pH 5.0 for 10 minutes. The plate was blotted with filter paper to remove excess buffer. The cellulose acetate was subjected to 7 minutes electrophoresis, which was then removed from the tank and immersed in 0.1 M barium acetate in 15% ethanol, pH5.0 for 3 minutes. The cellulose acetate plate was then blotted and replaced again in the tank to continue electrophoresis for a further 25 minutes. Cellulose acetate was taken out once again, immersed in 0.1 M barium acetate in 50% ethanol, pH5.0 for 3 minutes, blotted before replacing it back into the tank and continued with electrophoresis for 15 minutes. The electrophoresis banding pattern on cellulose acetate plate was visualized by staining with 5.0 g/L alcian blue 8GX for 20 minutes and destained with 1% v/v acetic acid twice for 15 minutes each time with gentle mixing. Cellulose acetate plate was left immersed overnight in sterile water for complete destaining. Cellulose acetate was then scanned to capture the freshly stained electrophoresis banding pattern for interpretation. The electrophoresis banding pattern was analyzed to determine the excretion GAGs and to characterize the isotypes of MPS.

RESULTS

We determined the detection limit of GAGs on cellulose acetate plate using the positive patient's sample (MPS II) as shown in Figure 1. Application volume of GAGs from the patient's sample was 2 μl, equivalent to approximately 120 mg of GAGs as this gave electrophoresis

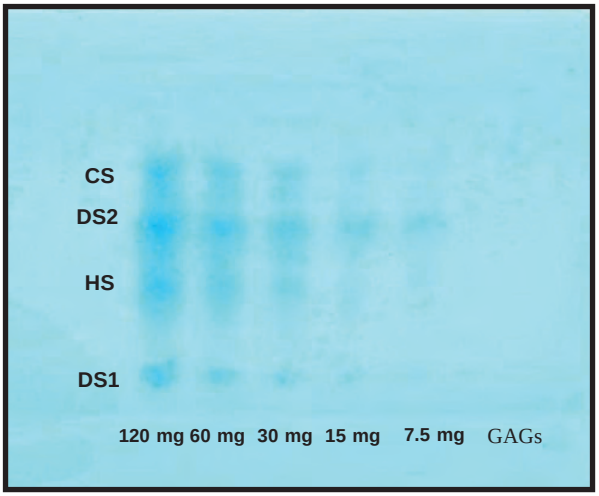

FIG. 1: Detection limit of GAGs on cellulose acetate plate high resolution electrophoresis. Electrophoretic pattern using five different amounts of urinary GAGs obtained from a positive MPS II control. The minimal GAGs amount required to give an interpretable electrophoretic pattern was 30 mg. CS: chondroitin sulphate; HS: heparin sulphate; DS: dermatan sulphate. DS was split into two bands (DS1 and DS2). 120 mg of urinary GAGs was used for all patients' samples because it gave electrophoretic pattern with high resolution.

TABLE 1: Clinical characteristics, urinary GAGs electrophoretic pattern and enzymatic diagnosis of 40 patients with MPS

Patient	Gender	Age (year)	Ethnic	Coarse facies	Skeletal abnormalities	Hapato-splenomegaly	Mental delay/ regression	Cornea opacities	Cardiac	Total GAGs/ mol Creatinine	Urinary GAGs HRE					HRE Pattern	Provisional diagnosis (MPS type)	Final diagnosis: deficient-enzyme (MPS type)
											KS	CS	DS2	HS	DS1			
1	F	11	I	++	++	+	+	+	-	61	-	+	-	++	++	A	IH	**α-L-iduronidase (MPS IH)**
2	M	5	Iii	+	+	+	+++	-	+	35	-	+	-	+++	-	B	III	**Sulphamidase (MPS IIIA)**
3	M	11	I	++	++	+	++	-	-	24	-	++	++	++	++	A	II	**Iduronate-2- sulphatase (MPS II)**
4	M	6	Iii	++	++	+	+	-	+	84	-	+	++	+	++	A	II	**Iduronate-2-sulphatase (MPS II)**
5	M	2	I	++	++	++	+++	-	-	100	-	++	++	-	++	A	II	**Iduronate-2-sulphatase (MPS II)**
6	M	5	I	++	++	+	++	-	-	43	-	++	++	++	++	A	II	**Iduronate-2-sulphatase (MPS II)**
7	M	4	i	++	++	+	+++	-	-	65	-	++	++	++	++	A	II	**Iduronate-2-sulphatase (MPS II)**
8	M	14	ii	+++	++	+	+++	-	+	38	-	+	+	+	++	A	II	**Iduronate-2-sulphatase (MPS II)**
9	M	9	i	+	+	+	+++	-	-	65	-	++	-	++	-	B	III	**Sulphamidase (MPS IIIA)**
10	M	5	i	+	+	+	+++	-	-	58	-	++	-	++	-	B	III	**Sulphamidase (MPS IIIA)**
11	F	2	ii	-	+++	-	-	+	-	36	+	+	-	-	-	C	IV	**N-acetylgalactosamine-6-sulphatase (MPS IVA)**
12	F	4	i	-	+++	-	-	-	-	34	+	+	-	-	-	C	IV	**N-acetylgalactosamine-6-sulphatase (MPS IVA)**
13	F	3	i	++	++	+	-	+	-	73	-	+	++	-	++	D	VI	**N-acetylgalactosamine 4 sulphatase (MPS VI)**
14	M	5	i	++	++	+	-	-	-	337	-	++	++	-	++	A	VI	**NA**
15	M	2	i	++	++	+	++	+	-	64	-	+++	+	+	+	A	II	**ß-glucuronidase (MPS VII)**
16	F	10	i	-	++	-	-	-	-	36	+	+	-	-	-	C	IV	**NA**
17	M	7	iii	-	+++	-	-	-	-	9	++	+++	-	+	-	C	IV	**NA**
18	F	7	i	-	+++	+	-	-	-	7	++	+++	-	+	-	C	IV	**NA**
19	M	5	iii	+	+	+	++	-	-	35	-	++	-	+++	-	B	III	**NA**
20	M	2	iv	++	+	+	++	-	-	50	-	+++	-	+++	-	B	III	**NA**

Patient	Gender	Age (year)	Ethnic	Coarse facies	Skeletal abnormalities	Hapato-splenomegaly	Mental delay/ regression	Cornea opacities	Cardiac	Total GAGs/ mol Creatinine	Urinary GAGs HRE					HRE Pattern	Provisional diagnosis (MPS type)	Final diagnosis: deficient-enzyme (MPS type)
											KS	CS	DS2	HS	DS1			
21	F	6	ii	+	+	+	++	-	-	32	-	++	-	++	-	B	III	**NA**
22	M	6	i	++	-	-	++	-	-	21	-	+	-	+	-	B	III	**NA**
23	F	2	i	+	-	+	+	-	-	43	-	++	-	++	-	B	III	**NA**
24	F	4	ii	+	+	-	++	-	-	46	-	+++	-	+	-	B	III	**NA**
25	M	10	i	+	+	-	++	-	-	21	-	++	-	+	-	B	III	**NA**
26	M	1	iv	+	+	+	++	-	-	47	-	+++	-	+	-	B	III	**NA**
27	M	7	iv	++	+	-	+++	-	-	32	-	++	-	+	-	B	III	**NA**
28	M	7	i	+	+	+	+++	-	-	31	-	++	-	+	-	B	III	**NA**
29	M	3	i	-	-	-	++	-	-	48	-	++	-	+	-	B	III	**NA**
30	M	4	i	-	-	-	++	-	-	37	-	+	-	+	-	B	III	**NA**
31	M	4	i	-	-	-	++	-	-	15	-	++	-	++	-	B	III	**NA**
32	M	9	i	+	-	-	++	-	-	29	-	++	-	+	-	B	III	**NA**
33	M	5	ii	++	+	+	+++	-	-	34	-	+	++	+	+	A	II	**NA**
34	M	3	ii	++	++	+	+++	-	+	59	-	+	++	+	+	A	II	**Iduronate-2-sulphatase (MPS II)**
35	M	5	i	++	++	+	+++	-	+	47	+	++	+	++		A	II	**Iduronate-2-sulphatase (MPS II)**
36	M	1	i	+	+	+	+	+	-	51	-	++	++	+	+++	A	II	**NA**
37	M	8	i	++	++	+	++	-	+	12	-	++	++	++	+++	A	II	**Iduronate-2-sulphatase (MPS II)**
38	M	2	iv	+	+	+	+	-	-	70	-	+	+	+	++	A	II	**NA**
39	M	5	ii	++	+	+	++	-	-	16	-	+++	+	+++	+++	A	II	**NA**
40	M	13	ii	+++	+	+	++	-	-	58	-	+	+	+	++	A	II	**NA**
Normal										**< 1 year <30, 1-4 years <19, 4-9 years <13, 9-17 years <9**								

Key: +++ =prominent, ++ = moderate, + = mild, - = absent, M = male, F = female, i = Malay, ii = Chinese, iii = Indian, iv = Other, NA = not available

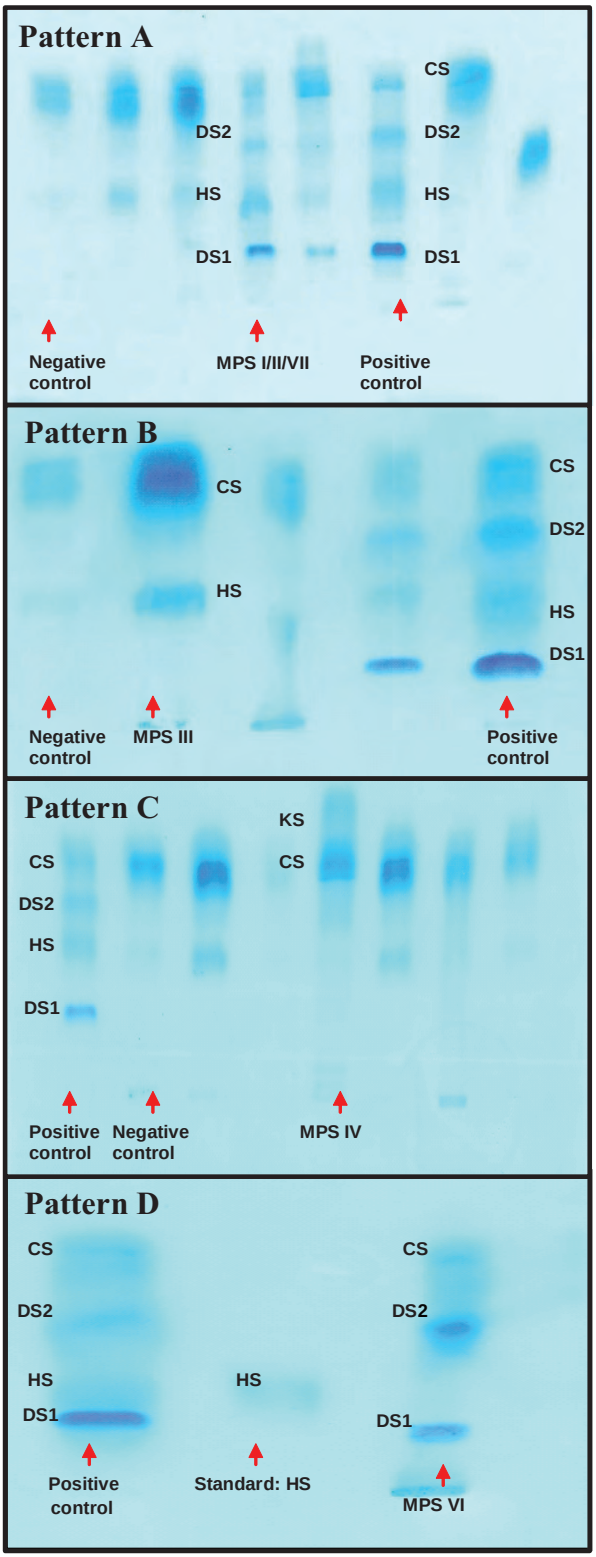

FIG. 2: Electrophoresis banding patterns of urinary GAGs. Abnormal HRE banding patterns could be classified into 4 patterns: Pattern A: elevated DS and HS which is suggestive of MPS I, II or VII. Pattern B: elevated HS and CS which is suggestive of MPS III. Pattern C: elevated KS and CS which is suggestive of MPS IV. Pattern D: elevated DS which is suggestive of MPS VI.

banding pattern with the highest resolution.

From 760 urine samples analyzed, 40 samples exhibited abnormal urinary GAGs HRE pattern. Clinical characteristics of these 40 cases and their electrophoresis banding patterns are summarized in Table 1. Seventeen cases have elevated DS and HS (HRE Pattern A or MPS I/II/VII); 16 cases have elevated HS and CS (HRE Pattern B or MPS III); five cases have elevated KS and CS (HRE Pattern C or MPS IV) while two cases has elevated DS only (HRE Pattern D or MPS VI) (Figure 2). From the 40 cases with abnormal HRE patterns, 35 of them also have elevated urinary GAGs level.

Of the 40 positive cases, 17 cases were investigated further with enzymatic studies (in an accredited commercial laboratory from Australia) according to the diagnostic flowchart as shown in Figure 3. These enzymatic studies revealed that 9 cases were confirmed to have MPS II, MPS I (1 case), MPS IIIA (3 cases), MPS IVA (2 cases), MPS VI (1 case) and MPS VII (1 case).

DISCUSSION

Our study was aimed to establish a simple, inexpensive and practical method to detect patients with MPS in Malaysia. We also aimed to differentiate various types of MPS so that the number of specific lysosomal enzymes needed to be studied could be minimized and be more affordable. With careful analysis of patients' urinary GAGs HRE banding patterns, we were able to give a provisional diagnosis of which MPS types the patient was suffering from. This was fairly straightforward for MPS III, MPS IV and MPS VI which gave HRE patterns distinct from other MPS types. On the other hand, HRE patterns of MPS I, II and VII were

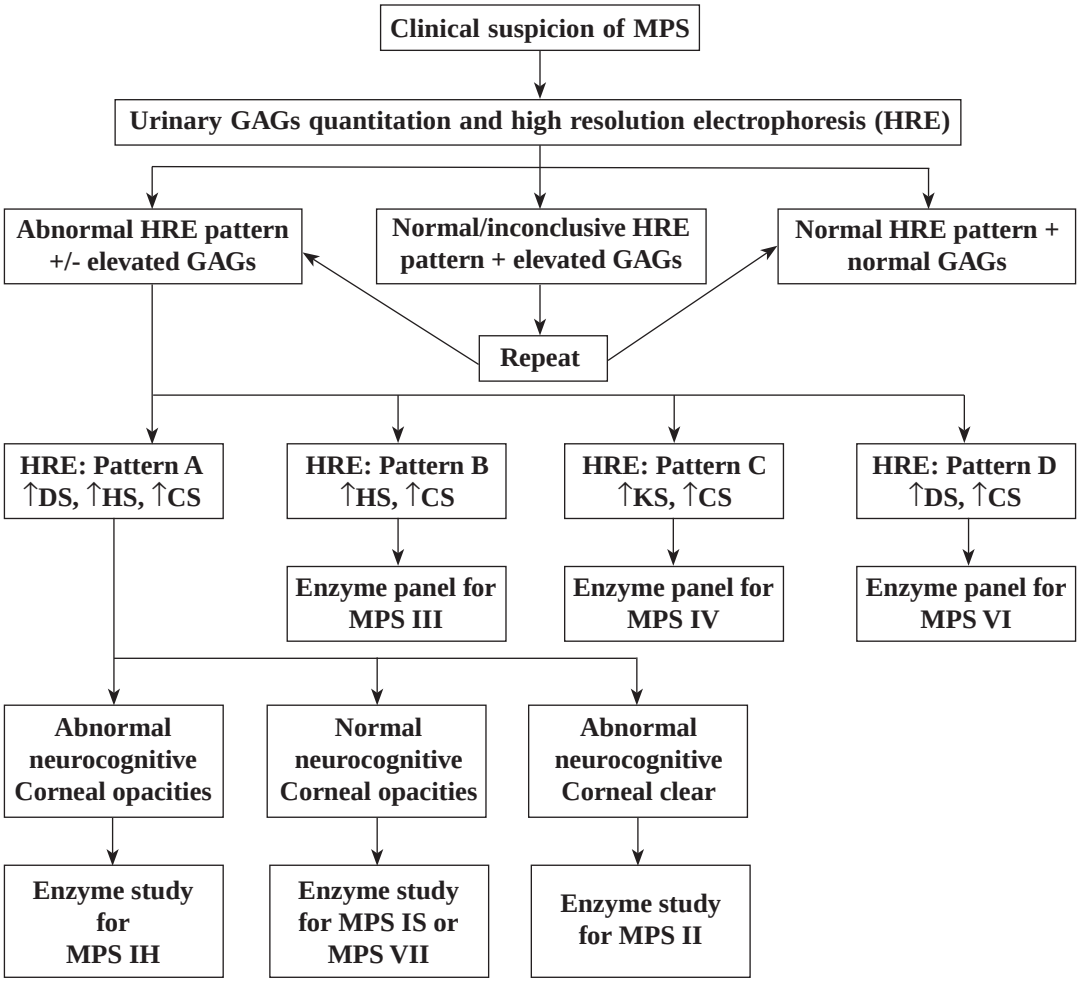

FIG. 3: Diagnostic flowchart for laboratory diagnosis of mucopolysaccharidoses.
Key: CS: chondroitin sulphate; HS: heparan sulphate; DS: dermatan sulphate; KS: keratan sulphate

indistinguishable from each other. However, by using a practical diagnostic flowchart which incorporated a few discriminative clinical signs and symptoms, we were able to select one or two lysosomal enzymes study to confirm the definite MPS type.

From the specific lysosomal enzymes study, we were able to confirm 17 cases out of the 40 cases identified as abnormal in the HRE screening. In most cases, we were able to arrive at the final diagnosis of MPS type by doing assay limited to one or two enzymes. Using the diagnostics flowchart, we were able to decide the probable enzyme or group of enzymes to be analyzed in most cases. However, this is contrary in exceptional cases such as in Patient 15 where enzyme activities were found to be normal for MPS I, II & VI. It is only with further enzyme analysis, that it was finally confirmed to be MPS VII which is an extremely rare MPS isotype.

Our finding also showed that five cases had normal urinary GAGs level but were shown to have abnormal urinary GAGs HRE patterns. This included one patient who was subsequently confirmed to be MPS II by enzymatic study. Thus, this shows that quantitation of total urinary GAGs alone is inadequate to screen for MPS. Urinary GAGs HRE is an essential component of the screening test for patients with suspected MPS. Gray G *et al* have made similar observation previously.[7]

Of the 17 patients in whom the definite lysosomal enzyme defects were known, MPS II appears to be more common than other MPS types. This may be an interesting observation as MPS I is the most common MPS type worldwide.[1] However, definite conclusion was not possible as we do not have enzyme assay for >50% of cases. In most cases, we did not have access to the patients' blood samples to perform the necessary enzyme assay in commercial laboratory.

CONCLUSION

In this study, we have shown the usefulness of HRE pattern in determining the provisional diagnosis of MPS. With this provisional diagnosis, specific enzymatic analysis can be identified. This is a less expensive test method and can reduce the cost for MPS diagnostics.

ACKNOWLEDGEMENT

We thank the Director General of Health Malaysia for permission to publish this paper. We would also like to express our gratitude to Lizaros Ahamad Sohor, Siti Hawa Musa, Mazanah Mohd Din, Rasfan Mat Lazin and all staff of Molecular Diagnostics and Protein Unit, Institute for Medical Research for their technical assistance. Our special thanks to Shahnaz Murad, Director of IMR and Rohani Md Yasin for critical reading of the manuscript and valuable comments. This research was supported by Small Research Grant, Institute for Medical Research (IMR).

REFERENCES

1. Wraith J. Mucopolysaccharidoses and Oligosaccharidoses. In: Fernandes J, Saudubray JM, van den Berghe G, Walter JH, editors. Inborn Metabolic Diseases. Heidelberg, Springer Medizin Verlag; 2006. p. 495-507.
2. Blau N, Hoffmann GE, Leonard J, Clarke JTR. The mucopolysaccharidoses. In: Schöder G, editor. Physician's guide to the laboratory diagnosis of metabolic diseases. Heidelberg, Springer; 2006. p. 195–203.
3. Giugliani R, Harmatz P, Wraith JE. Management guidelines for mucopolysaccharidosis VI. Pediatrics 2007; 120: 405–18.
4. Andrade F, Prieto JA, Elorz J, *et al*. Stablity of urinary glycosaminoglycans in patients with mucopolysaccharidoses. Clin Chim Acta. 2008; 388: 73–7
5. Hopwood JJ, Harrison JR. High-resolution electrophoresis of urinary glycosaminoglycans: An improved screening test for the mucopolysaccharidoses. Anal Biochem. 1982; 119: 120-7.
6. Cappelletti R, DelRosso M, Chiarugi VPA. New electrophoretic method for the complete separation of all known animal glycosaminoglycans in a monodimensional run. Anal Biochem. 1979; 99: 311-5.
7. Gray G, Claridge P, Jenkinson L, *et al*. Quantitation of urinary glycoaminoglycans using dimethylene blue as a screening technique for the diagnosis of mucopolysaccharidoses – an evaluation. Ann Clin Biochem. 2007; 44: 360-3.