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ORIGINAL ARTICLE

Diagnosis of Major Organic Acidurias in Children: Two Years Experience at a Tertiary Care Centre

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Abstract Organic acid disorders are inherited metabolic disorders in which organic acids accumulate in tissues and biological fluids of affected individuals. Classical organic acidurias include methylmalonic aciduria, propionic aciduria, isovaleric aciduria and maple syrup urine disease (MSUD). They are considered the most frequent metabolic disorders among severely ill children. Patients frequently present with acute symptoms early in life. 420 cases clinically suspected to have organic aciduria, with upper age limit of 12 years for a 2-year period (January 2007–December 2008) were enrolled into this study. Metabolic acidosis and neurological symptoms were the most common signs. Screening tests and thin layer chromatography were done for detection of organic acidurias. Identification and quantitation of organic acids in urine and quantification of amino acids in blood were done by high performance liquid chromatography. Out of 420 patients, 45 patients (10.7%) were found to have organic acidurias. 15 cases of methylmalonic aciduria, 16 cases of propionic aciduria, 13 cases of MSUD, and one case of isovaleric aciduria were diagnosed. Results demonstrate the importance of testing for organic acidurias. Since organic aciduria may cause irreversible brain damage if not treated, we recommend selective screening amongst severely ill children despite implied extra costs.

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Introduction

Newborn screening is highly relevant from socio-economic aspects as undetected cases of metabolic disorders lead to permanent mental retardation [1]. About 1/3rd of pediatric mental retardation cases may be attributed to the inability to detect a metabolic disorder in early childhood. Newborn screening is aimed at early detection and intervention of treatable inborn errors of metabolism and also to establish incidence of these disorders in this part of the world [2]. Given the density of population, the rate of occurrence may be expected to be high and preventable complications can be attended to early and before the affected child is incapacitated [3].

It may not be viable economically and ethically to screen for a complete range of disorders for which diagnostic modalities are available. Wilson JMG [4] have outlined specific criteria that serve as a template to decide what disorders to include in the screening at a national platform. These are: (*a*) biochemically well identified disorder; (*b*) known incidence in the population; (*c*) disorder associated with significant morbidity and mortality; (*d*) effective treatment available; (*e*) period before which intervention improves outcome; and (*f*) availability of an ethical, safe, simple and robust screening test.

Organic acidurias (OAs) arise due to alterations of the intermediary metabolism that lead to the accumulation of organic acids in the tissues, acid–base imbalance and intracellular biochemical alterations [5]. Organic acid disorders (OAD) are considered the most frequent inherited

metabolic disorders among severely ill children [6–8], and together with the aminoacidopathies constitute the most prevalent groups of inborn errors of metabolism in high-risk populations [9]. The range of clinical manifestations in organic acidurias is extensive, involving multiple body systems with predominance of the central nervous system [10].

The diagnosis of these diseases requires identification of abnormal pattern of organic acids in biological fluids especially urine, by gas chromatography/high performance liquid chromatography (HPLC). Because these procedures require expensive equipment and qualified professionals, diagnosis of organic acidemias is generally available only at a few regional reference centers. Effective treatment for many organic acidemias has improved during the last decade, a fact that renders their prompt diagnosis worthwhile. The prevalence of organic acidurias is possibly high in our part of the world [11]. This study has been therefore undertaken to find out the prevalence of organic acidurias in the study population.

Materials and Methods

Study was performed in 420 consecutive high-risk children from Amrita Institute of Medical Sciences with suspicion of inborn errors of metabolism (IEM) especially organic acidemia from January 2007 to December 2008. During the period the total population we screened for IEM was 4899 cases. Upper age limit was 12 years. Parents or guardians, signed informed consent forms for collection of the biological samples for laboratory analysis. Written informed consent was obtained from all study subjects. The study protocol conforms to the ethical guidelines of the "World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo 2004. The study was approved by the institutional ethical review committee. Fresh random urine sample (20 ml) was collected without preservative. A blood specimen was also collected with EDTA as the anticoagulant, the plasma immediately separated. Presenting features and age at diagnosis and presentation, as well as sex, parental consanguinity, family history of IEM and unexpected deaths were recorded.

All urine samples were initially screened by qualitative tests for inborn errors of metabolism. Rothera's test, dinitrophenyl hydrazine (DNPH) test, ferric chloride test, ninhydrin test, Benedict's test, glucose oxidase test, test for homogentisic acid, test for urea cycle metabolites, Ehrlich's test as well as thin layer chromatography for amino acid and para nitro aniline test for methylmalonic acid on urine samples were done. Figure 1 shows the main steps in

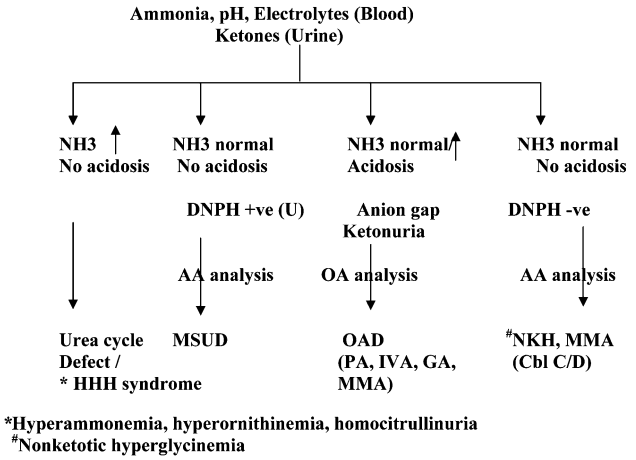

Fig. 1 Flow chart for diagnosis of organic acidurias

investigation of organic acidurias. Chromatographic analysis of urine (organic acids, carbohydrates or amino acids) [12, 13] and quantitative assays for organic acids and amino acids by HPLC [14] were carried out following previously published protocols, when at least one screening test showed a positive or doubtful result or when a strong clinical suspicion existed. The positive samples from these screening tests were submitted for urine organic acid quantification by HPLC. In suspected cases of amino acid disorders, plasma amino acid analysis was performed by HPLC analysis on blood specimens. In Methylmalonic aciduria (MMA) patients, serum vitamin B_{12} was estimated by chemiluminescence method.

HPLC Analysis Conditions for Organic Acids

The mobile phase for the chromatography was potassium dihydrogen orthophosphate buffer, adjusted to pH of 3.5. The flow rate for mobile phase was 1 ml/min. We used a 4.6 mm × 250 cm column of phenomenex Lichrosorb C-18. Detection was carried out at 206 nm at a temperature of 25°C using PDA detector.

1 ml of urine and 1 ml of acetone were taken in a test tube, vortex mixed and centrifuged. To the supernatant, premixed desalting solution A and B [Desalting solution A-$ZnSO_4$, Desalting solution B-Ba $(OH)_2$] was added, vortex mixed and centrifuged. The supernatant was filtered with Whatmann No. 1 filter paper. 20 µl of the filtrate was injected into the chromatograph. 20 µl of 0.4234 mmol/l (50 ppm) methylmalonic acid, 1.012 mmol/l (75 ppm) propionic acid and 1.958 mmol/l (200 ppm) isovaleric acid standards were used for quantification. HPLC separation was performed with isocratic elution at a flow rate of 1 ml/min, at 25°C and 206 nm absorption wavelengths. Organic acids were detected based on the retention time established for the individual organic acids under defined experimental

conditions. Linearity of the peak areas for different concentrations, ranging from 0.125 to 0.625 μmoles of individual organic acids was determined. Calculation was based on the area under peak established for a given organic acids of known standard concentration. The final results were expressed as mmol/l [15].

HPLC Analysis Conditions for Amino Acids

HPLC system consists of Shimadzu LC-10AT VP solvent delivery system, a manual injector with a six-port injection loop, a Diode array detector SPD-M10A VP and a column oven CTO-10AS VP. The compounds were separated by gradient elution on a Phenomenex Luna C18 column (250 × 4.6 mm, 5 micron) with a guard column (Phenomenex 4.0 × 3.0 mm packed with reversed phase C18 material) at 40°C. The mobile phase consists of a sodium phosphate buffer of pH 7.0 and acetonitrile. Photo Diode Array (PDA) detection was performed at 254 nm.

400 μl plasma was mixed with 200 μl 10% sulphosalicylic acid at 4°C for 30 min and centrifuged. 100 μl 100 mM phenylisothiocyanate (PITC) and 100 μl 1 M Triethyl amine (TEA) were added to 200 μl of the supernatant or standard, and allowed to stand at room temperature for 2 h. 400 μl hexane was mixed with 200 μl of PITC-TEA- supernatant mixture and vortex mixed. After separating the hexane layer, 20 μl membrane filtered (0.45 μm) aqueous layer was injected to HPLC system.

Amino acids were separated using mobile phase 10 mM phospate buffer, pH 7.0 (solvent A) and acetonitrile (solvent B), and gradient was programmed using concentration of B as 5% (0 min), 35% (28 min), 80% (28.01 min), 80% (33 min), 5% (33.01 min) and 5% (39 min). 20 μl of 0.625 μmol/ml (except cystine 0.3125 μmol/ml) of amino acid standard was used for quantification of the different amino acids. HPLC separation was performed with gradient elution at a flow rate of 1 ml/min, at 40°C and PDA detection was performed at 254 nm absorption wavelength. Amino acids were detected based on the retention time established for the individual amino acid under defined experimental conditions. Linearity of the peak areas for different concentrations, ranging from 0.125 to 0.625 μmol of individual amino acids was determined. Calculation was based on the area under peak established for a given amino acid of known standard concentration. The final results were expressed as mmol/l [16].

Results

We analyzed urine samples from 420 children and identified 45 children with consistent (at least on two occasions) altered pattern of organic acid excretion. We have detected 45 cases (10.7%) of organic acidemias in 420 individuals tested by organic acid analysis. Among them 179 (42.6%) were females and 241 (57.4%) were males. The most frequent disorders in our high-risk population were propionic aciduria (16 cases, 3.8%) and methylmalonic aciduria (15 cases, 3.5%). Out of the 15 cases that showed methylmalonic aciduria, 7 cases showed vitamin B_{12} deficiency. We identified 13 cases (3.0%) of maple syrup urine disease and one case of isovaleric aciduria (Table 1). Average age at diagnosis was 24 months, and mean age at presentation was 5 months. Interestingly hyperglycinuria without hyperglycinemia observed in the semi quantitative thin layer chromatography, led us to investigate organic acid disorders by HPLC, giving rise to 20 cases of organic acidurias.

Table 2 shows the clinical and laboratory features of patients with organic acidurias. It can be seen that neurological abnormalities (60%) were the most frequent presenting feature of these patients, followed by metabolic acidosis (42%), hyperammonemia (30%), feeding difficulties (26%), hypoglycemia (26%), vomiting (25%), skin rashes (20%), lactic acidemia (20%), hepatomegaly (16%), failure to thrive (16%), hypo/hyper/dystonia (12%), ketosis/ketonuria (8%) and dysmorphies (6%).

Table 3 shows the major neurological features and Magnetic Resonance Image (MRI) abnormalities of these patients. Psychomotor delay/mental retardation (55.5%), seizures (40%), hypo/hyper/dystonia (46.6%), coma (15.5%), ataxia (11.1%), lethargy (24.6%), encephalitis/encephalopathy (6.6%), extra pyramidal syndrome (2.2%), visual deficiency (2.2%), speech delay (1.1%), hyperactivity (1.1%), were the major neurological signs. Abnormal MRI findings were found in 11 patients. Macrocephaly (15.5%), cerebral atrophy

Table 1 Organic acidurias diagnosed in high risk patients ($N = 420$)

Disease	Positive cases	Percentage of cases	Avg. age of presentation (m)	Avg.age of diagnosis (m)
Methyl malonic aciduria	15	3.5	5	24
Propionic aciduria	16	3.8	5	25
Maple syrup urine disease	13	3.0	6	24
Isovaleric aciduria	1	0.2	6	24
Total	45	10.7	6	24

Table 2 Major clinical and laboratory findings at diagnosis in patients with OAD ($N = 45$)

Clinical features	Percentage of patients
Neurological abnormalities	60
Hypo/hyper/dystonia	12
Vomiting	25
Feeding difficulties	26
Hepatomegaly	16
Failure to thrive	16
Dysmorphies	6
Skin rashes	20
Laboratory findings	
Metabolic acidosis	42
Hypoglycemia	26
Lactic acidemia	20
Hyperammonemia	30
Ketosis/ketonuria	8

Table 3 Neurological abnormalities in patients with OAD ($N = 45$)

Features	Number	Percentage
Neurological signs		
Psychomotor delay/mental retardation	25	55.5
Seizures	18	40
Hypo/hyper/dystonia	21	46.6
Coma	7	15.5
Ataxia	5	11.1
Lethargy	11	24.4
Encephalitis/encephalopathy	3	6.6
Extra pyramidal syndrome	1	2.2
Visual deficiency	1	2.2
Speech delay	5	11.1
Hyperactivity	5	11.1
Abnormalities on cerebral MRI		
Cerebral atrophy	3	6.6
Macrocephaly	7	15.5
Cerebral edema	1	2.2

(6.6%) and cerebral edema (2.2%) were the major MRI changes, among those detected to be abnormal. MRI abnormalities in brainstem and periventricular white matter in a 4 month aged patient from this study are shown respectively, in Figs. 2 and 3. Abnormal levels of branched chain amino acids, methylmalonic acid and propionic acids are shown in Figs. 4, 5 and 6.

Table 4 compares the frequency of OAD detected in our laboratory in high-risk patients with studies performed in other populations [25–28, 30, 31]. The frequency of these

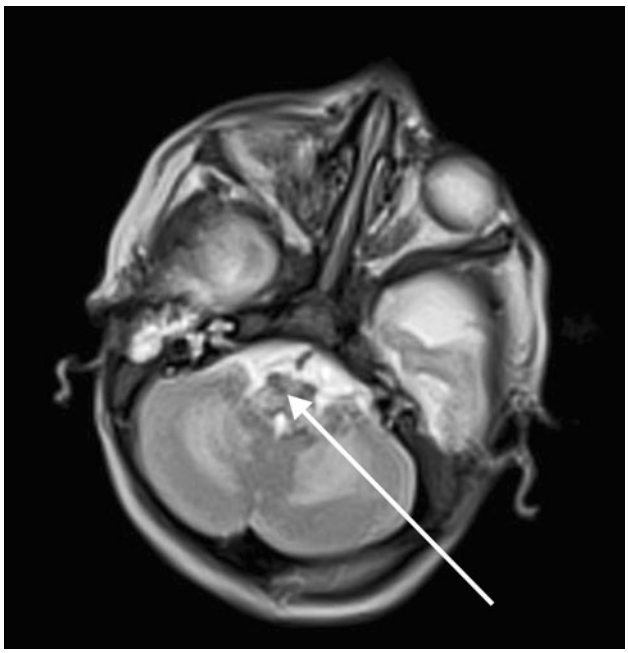

Fig. 2 MRI: Axial T2 W MRI demonstrating hyperintensity involving brain stem

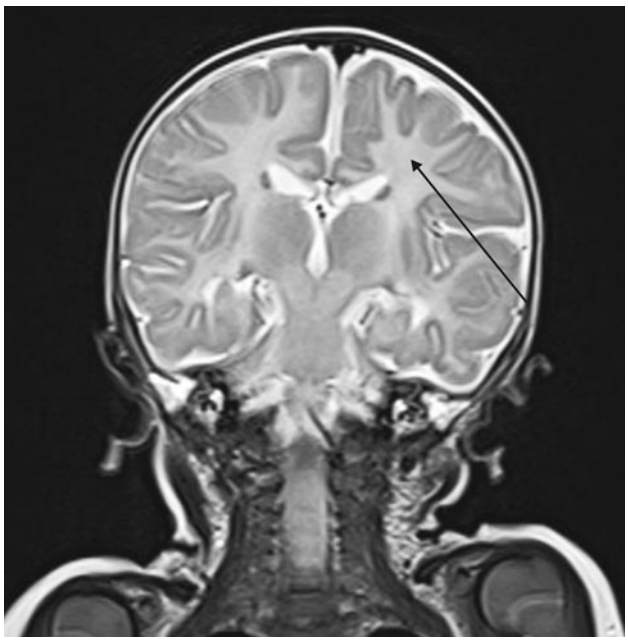

Fig. 3 MRI: Coronal T2 W MRI demonstrating hyperintensity involving periventricular white matter

disorders found in our population was similar to that found in other countries.

Average age of clinical presentation was 6 months and mean age of diagnosis was 24 months, which could be attributed to lack of awareness of these genetic diseases among physicians and/or to lack of laboratory services in

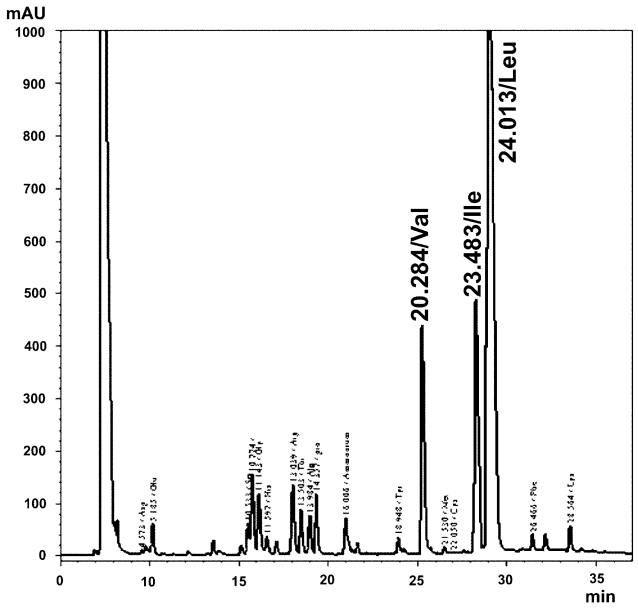

Fig. 4 The elevated levels of branched chain aminoacids in MSUD patient

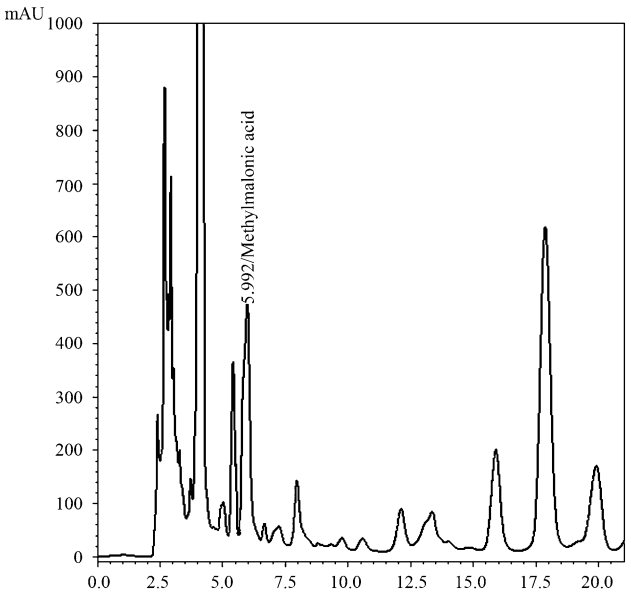

Fig. 5 The elevated levels of methylmalonic acid in methylmalonic aciduria patient

diagnosis of these disorders, high mortality of patients affected by these diseases or a combination of these factors. In 14 cases (31.1%), there was family history of past neonatal or infant death, and first-degree consanguinity occurred in 16 (35%) cases of the patients.

Considering the outcome of patients diagnosed and treated at our hospital, Amrita Institute of Medical Sciences and Research Centre, records obtained from 30 patients showed that all are under therapeutic regimens

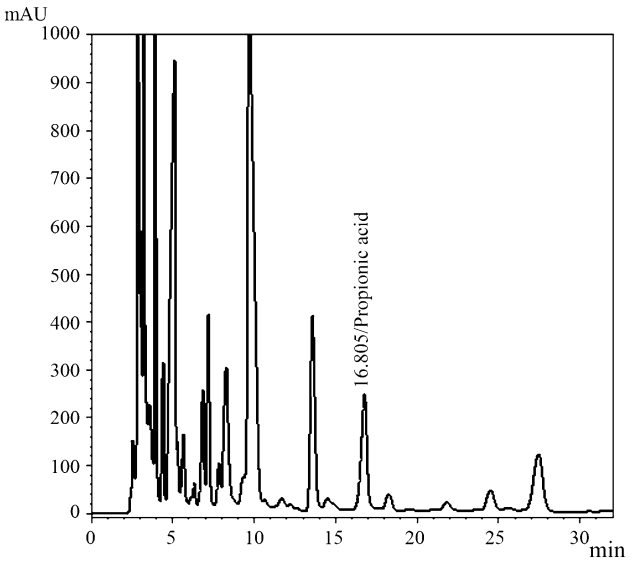

Fig. 6 The elevated level of propionic acid in propionic aciduria patient

based on protein restriction (1.5–2.0 g protein/kg/day), sodium bicarbonate (to correct acidosis), L-carnitine, and vitamins. Prompt diagnosis allowed specific treatment in the majority of these patients with rapid improvement in symptomatology in 20 and moderate improvement in 10, indicating the importance of diagnosing these diseases even after the symptoms onset by selective screening. Patients who responded best to treatment were those affected with methylmalonic acidemia and propionic acidemia, whereas children affected by maple syrup urine disease improved in symptomatology but not as effectively.

Discussion

The present report is a complete study on the diagnosis of common disorders of organic acid metabolism in high-risk patients using HPLC analysis over 2 years. A total of 420 consecutive symptomatic patients with suspicion of IEM especially of OAs had their urine specimens analyzed for the pattern of organic acids. Blood and urine specimens from these patients were submitted to screening tests for the detection of a wide spectrum of IEM. We found 45 cases of organic aciduria (10.7%) in this study population. Among them the most frequent disorders were methylmalonic aciduria (15 cases, 3.5%) and propionic aciduria (16 cases, 3.8%). We identified 13 cases (3.0%) of maple syrup urine disease and one case of isovaleric aciduria. In patients with typical presenting complaints, clinical findings, neuroimaging/initial biochemical test findings and the results of urine and blood HPLC analysis help in confirming the diagnosis.

Table 4 Organic acidemias detected in different populations

Disorders	Riyadh (3 years)	Paris (20 years)	San Diego (2 years)	Freiburg (18 years)	Asia (8 years)	Singapore (13 years)	India (2 years)
No. of patients	76	77	41	90	111	24	45
Methylmalonic aciduria	31 (41%)	31 (40%)	20 (48%)	34 (37%)	74 (67%)	7 (29%)	15 (33%)
Propionic aciduria	30 (39%)	21 (27%)	21 (51%)	33 (36.6%)	23 (20%)	8 (33%)	16 (36%)
Glutaric aciduria type I	10 (13%)	11 (14%)	ND	7 (8%)	6 (5%)	5 (21%)	ND
Isovaleric aciduria	5 (7%)	14 (18%)	ND	16 (18%)	1 (0.9%)	ND	1 (2.2%)
Maple syrup urine disease	ND	ND	ND	ND	7 (6%)	4 (17%)	13 (29%)

The data were obtained from several studies including Saudi Arabia, France, USA, Asia, Singapore and our present data (India): Riyadh [26]; Paris [27] San Diego [28]; Freiburg [25]; Asia [31]; Singapore [30]

ND not detected

In a hospital based study in India biochemical screening of 4,400 cases of mental retardation revealed that 256 (5.75%) cases were due to metabolic disorders [17–19]. Other studies from South India reported the presence of disorders of amino acid metabolism among 0.66–2.4% of children with mental retardation. [20–23] 1,094 (42.7%) out of 2,560 subjects screened for amino acid metabolism had mental retardation while 579 (22.6%) was infants [24]. In our study 25(55.5%) of organic aciduria patients were mentally retarded (Table 3). Mental retardation, seizures and tone abnormalities were the predominant features of organic acidemias. 15 patients died following an acute crisis of metabolic acidosis associated with convulsions, respiratory distress, coma or cardiac arrest, during the last follow-up. Abnormal MRI findings were found in 11 patients. MRI abnormalities have been described in organic acid disorders including white matter changes in MSUD and abnormalities of the globus pallidus in MMA [25].

High incidence was obtained for methylmalonic acidemia and propionic acidemia, considered the most prevalent single organic acidurias [26–29]. Table 4 shows the comparison of our results with other reports. Study conducted by Mamta et al. (2001) on the incidence of organic acidurias in India found out that out of 365 patients with IEM diagnosed over a period of 20 years, organic aciduria accounted for 27% of cases (MMA 18%, PA 9.2%) [30]. Study conducted by It-Koon Tan et al. (2006) revealed high incidence of organic acidurias [31]. The overall detection of organic acidurias revealed by Daisuke Hori et al. (2005) was 3.1% comprising mainly MMA and PA i.e, the predominance of MMA over PA was seen in Asian children [32]. MMA is the most common organic acidemia in many studies [32, 33]. Death occurred in at least 15 patients (33.3%) and in those, 8 cases diagnosis was performed very late, so that treatment could not be instituted. In this context, the natural course of symptomatic patients with OAs is often severe and when a late diagnosis is performed the outcome becomes worser. The relatively high mortality of our patients may be possibly explained by the gap between age at presentation and age at diagnosis in our sample.

Conclusion

Our results indicate the importance of diagnosing organic acidemias in developing countries particularly in severely ill patients. Availability of therapy for many of these disorders and treatment control of affected individuals by serial analysis of urine and blood is also important and appears additionally to justify the setup of such facilities despite implied extra costs.

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