ORIGINAL ARTICLE ASIAN JOURNAL OF MEDICAL SCIENCES

Screening for aminoacidopathies using cost effective methods in symptomatic children: A cross sectional study

Nazmin Fatima[1], Gyanendra Kumar Sonkar[2], Shalini Tripathi[3], Sangeeta Singh[4], Abbas Ai Mahdi[5]

[1]Research Associate, [2]Associate Professor, [4]UGC Women Postdoctoral Fellow, [5]Professor & Head, Department of Biochemistry, King George's Medical University, U.P., Lucknow-226003, India, [3]Associate Professor, Department of Pediatrics, King George's Medical University, U.P., Lucknow-226003, India

Submission: 25-09-2020 Revision: 29-12-2020 Publication: 01-02-2021

ABSTRACT

Background: Inborn errors of metabolism (IEM) are a cluster of hereditary disorders which are caused due to defect in one or more enzymes or disturbance in the protein transport system. **Aims and Objective:** The aim of this work was to find out the frequency of aminoacidopathies among symptomatic children and to emphasis about cost effective screening of aminoacidopathies in order to minimize morbidity and mortality. **Materials and Methods:** This cross-sectional study recruited 264 symptomatic children from infants to 15 years of age. Random urine samples (15ml) in sterile container and 1-2 ml of blood in EDTA vials were collected and stored at -20˚C. Color reactions, TLC and HPLC were done in urine and blood samples for amino acid disorders. **Results:** The prevalence of aminoacidopathies was found high in early age (45.1%), males (55.3%), rural areas (75%) and Muslims (39%). A total of 127 out of 264 (48.1%) symptomatic cases showed at least one of the positive colored tests. Mean hemoglobin level was lower than the normal reference range. 7.7% children had severe anemia (Hb < 7 g/dl). TLC of all these 127 samples was done for amino acids. The results showed positive elevated amino acids in 61 cases (48%). Out of 61 cases only 29 cases were positive for amino acid disorders by HPLC. Generalized aminoaciduria was the common followed by MSUD and tyrosinuria. Based on combination of these three tests, we were able to detect 10.98% (29/264) cases of aminoacidopathies. **Conclusions:** Hence this study focused using a set of rapid, easy and cost effective simple tests for screening in symptomatic children. There is need to increase the awareness in developing country like India regarding the availability of such affordable and reliable tests.

Key words: Inborn errors of metabolism; Aminoacidosis; HPLC; TLC; Aminoacidopathies

Access this article online

Website: http://nepjol.info/index.php/AJMS

DOI: 10.3126/ajms.v12i2.31497

E-ISSN: 2091-0576

P-ISSN: 2467-9100

INTRODUCTION

Inborn errors of metabolism (IEM) are a cluster of hereditary disorders which are caused due to defect in one or more enzymes or disturbance in the protein transport system. The abnormal enzyme disrupts the metabolic process and leads to deficiency of products which are essential for cell function, accumulation of substrates or products of the alternative pathway. IEMs are individually unusual with the incidence of one in one lakh. But because of the enormous enzymatic derangement, they are collectively common with an overall incidence of one in 800 to one in 2,500. They are the greatest contributors to the incurable disease in childhood.[1-2]

One single test is not enough to identify the IEM. Different strategies of diagnosis are in use to detect it. Accumulated metabolites can be estimated in blood or urine. To improve the establishment of diagnosis and management of long term consequences, newborn screening (NBS), gas

Address for Correspondence:
Dr. Gyanendra Kumar Sonkar, Associate Professor, Department of Biochemistry, King George's Medical University, Lucknow- 226003, India. **Mobile No:** +91-9453817641. **E-mail:** gyanendrakrsonkar@kgmcindia.edu

chromatography-mass spectrometry (GCMS), tandem mass spectrometry (TMS), molecular analysis, and enzyme assay have been developed. The NBS is widely implemented in developed countries like the USA, Japan, and a few European countries. In contrast to this, less information is available from the developing countries,[3,4] including India. A study conducted by Verma et al. (2006) has revealed that about 0.08% of newborns and 3.2 % of high-risk children are affected by metabolic disorders in India.[5]

The prevalence of IEM varies from one country to another country and region to region in the same country. Many factors are responsible for IEM such as race, consanguinity, genetic makeup, environment, feeding habits, and way of life of individuals.[6,7] Advanced technologies like GCMS, HPLC, and TMS are used in developed countries.[8] Urine metabolic screening, which includes color reactions and TLC, requires only minimal laboratory equipment and can be conducted easily in any laboratory. There is a need to create these basic facilities which include simple diagnostic screening tests in each district hospital before referring to the higher centers. We have attempted to study the usefulness of noninvasive urinary metabolic screening using color reactions, TLC, and high-performance liquid chromatography (HPLC) of amino acids abnormalities in symptomatic children. Hence, these tests are of great importance to screen cases in small laboratories and in the primary health centers where high and sophisticated tests are not available. Hence the study was undertaken to estimate the spectrum and the magnitude of suspected IEM in our tertiary care center with the help of clinical symptoms and basic metabolic workup which are not only simple but also cost-effective.

MATERIALS AND METHODS

The target population in this study comprised children clinically suspected for IEM having a history of lethargy, poor feeding, recurrent vomiting, convulsion, unusual odor, consanguinity, hypoglycemia, hepatic failure, poor growth, developmental delay, regression of milestone, failure to thrive, coarse facial, mental retardation, skeletal deformities, sibling death with a similar illness, progressive disease with intermittent or continuous loss of skills. Children between infancy to 15 years of age were recruited. We divided these children into three groups i.e. group A comprising of children between 1 month to 1 year, group B and C included 1.1 to 5 years and 5.1 to 15 years respectively.

The study was conducted in the Department of Biochemistry in collaboration with the Department of Pediatrics at King George's Medical University, Lucknow. Written informed consent was taken from parents/guardians. The study was approved by the Ethical Committee of the Institution. All procedures performed in this study were in accordance with the ethical standards of this institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Laboratory investigation

Complete blood count (CBC), Arterial blood gas (ABG), blood pH, blood urea, serum creatinine, total protein, total albumin, serum electrolytes, blood glucose, liver function tests (LFT) and kidney function tests (KFT) were done with commercially available ortho-clinical diagnostic kits (Johnson and Johnson) using Vitros 250 system chemistry auto-analyzer.

Sample preparation

Random urine samples (15ml) were collected from subjects and labeled with an identification number. Urine samples were centrifuged at 1000 rpm for 10 minutes and the supernatant was separated for further analysis. 1-2 ml of blood in EDTA vials was centrifuged and plasma was separated and stored at -20°C.

Color reactions, such as Benedict's test, Seliwanoff's test, Rothera's test, ferric chloride test, dinitrophenyl hydrazine test (DNPH), nitrosonaphthol test and homogentisic acid test were performed in urine samples. All reagents used for the tests were from Sigma (St. Louis, Missouri

United States), Merck (Kenilworth, New Jersey, United States), SRL (New Delhi, India), and Himedia (Mumbai, India).

Thin layer chromatography

TLC for amino acids was performed in all urine and blood samples using a solvent mixture of normal butanol, acetic acid, and water in the ratio 12:3:5 by volume. 2% Ninhydrin working reagent in acetone was used for staining.

HPLC

HPLC (Agilent 1260) was used for the quantitative estimation of amino acids in blood & urine samples. The online derivatization was performed using ortho-phthaldehyde (OPA) and 9-fluorenylmethyloxycarbonyl (FMOC). Mobile phase A (10 mM Na_2HPO_4, 10mM $Na_2B_4O_7$ & NaN_3) mobile phase B (45% Acetonitrile, 45% Methanol and 10% Water) of pH 8.2 was used for elution and analysis of amino acids.

Data analysis

Sociodemographic and clinical characteristics including biochemical parameters, urine analysis using colored reactions, TLC and HPLC were tabulated and analysed using IBM SPSS version 24 software. Descriptive statistics were expressed in numbers, percentages, and mean ± SD.

Quantitative data were analysed using independent t-test and one way ANOVA.

RESULTS

Demographic data and clinical manifestation in symptomatic children

It shows an association of familial characteristics with religion and it was found that nearly half the Muslim subjects (52/103) had significant high frequency of consanguinity (p<0.001). (Table 1).

It was observed that there was no significant association of familial characteristics with region of inhabitance (Table 2).

Upon analyzing of biochemical parameters with consanguinity (Table 3) mean of hemoglobin level was significantly decreased in consanguineous as compared to non-consanguineous group.

Clinical manifestations were more pronounced in early age. About 55.3% had symptoms in the very first year of life. Development delay was observed in 47 cases, out of which 34 (72.3%) were in the age group of infant to 5 years. Upon combining two group i.e. group A and group B, lethargy, recurrent vomiting, convulsion, jaundice and hypoglycemia were found 21.4%, 17.8%, 12.2%, 11.7% and 11.7% respectively (Table 4).

In Table 5, total protein and albumin were low in group A while total and direct bilirubin was higher in group A as compared to rest of the two groups. Blood sugar showed increasing trend of mean level with age. Serum ALT and AST were high in group A as compared to the rest two groups.

Screening of IEM in symptomatic children using color reactions, TLC and HPLC

Cost effective urinalysis was performed by color reactions for amino acids in all the 274 cases. The flow chart of distribution of cases is shown in Figure 1. This figure shows the number of positive cases obtained using various tests such as color reactions, TLC and HPLC.

Color reaction

A total of 127 out of 264 (48.1%) cases showed at least one of the positive colored tests as mentioned in the methodology section.

It was observed that more number of cases showed positive colored reactions in group A followed by group B and group C (44.1% versus 28.3% versus 20.5% respectively). Combining the first two age groups it was found that positive colored reactions was obtained in 72.4% cases. Positive cases are seen more in early stage of life, hence early detection is advised (Table 6).

Detection of amino acid by TLC

Next, TLC of all these 127 positive samples (by color reactions) was done for amino acids in urine samples. The results obtained showed positive elevated amino acids and spots in 61 cases (49.6%).

The results shows that TLC positive samples (34/61, 53.9%) were higher in group A, followed by that in group C (16/61) and group B (13/61) (25.4% and 20.6% respectively). The table also shows that out of 13 cases of branched chain amino acids, 7 cases (53.8%) were in group A. However their confirmatory test could be done by HPLC. 19 children (19/61, 30.1%) had history of consanguinity (Table 7).

Finally, all those samples which showed positive amino acid spots in TLC test were subjected for HPLC analysis. Among these 61 samples, we found 29 samples (47.5%) with abnormally high amino acids level when compared with normal range for respective age group(Table 8). In this study all those cases were considered to be positive in HPLC analysis when their urine or plasma level was found to be at least twice or more elevated than the maximum limit of their normal levels.

Based on combination of three tests i.e. colored reactions, TLC and HPLC, we were able to detect 10.98% (29/264) cases of aminoacidopathies among all the symptomatic cases recruited for the study. The screening of samples with color reactions and TLC yielded 23.1% (61/264) aminoacidopathies. Nearly half the samples i.e. 49.6% among 127 cases showed positive colored spots in TLC. Similarly, 29/127 (22.8%) cases were of aminoacidopathies which accounts for nearly one fifth of total positive colored reactions.

DISCUSSION

Screening for IEM is a routine practice in many developed countries like US, Europe, Japan and other countries. Simple

Table 1: Association of familial characteristics with religion and region

S.No.	Familial characteristics	Religion			P value
		Hindus(149) N (%)	Muslims(103) N (%)	Others(12) N (%)	
1.	Consanguinity	18 (12.1)	52 (50.5)	3 (25.0)	<0.001*
2.	Sibling illness/deaths	20 (13.4)	15 (14.5)	1 (8.3)	0.83
3.	Family history	5 (3.4)	10 (9.7)	0 (0.0)	0.06

and cost-effective techniques can be helpful in picking up cases suspected for IEM. In India the occurrence of IEM is not exactly known. This may be due to the lack of studies on IEM based in a large population. Few reports from South India are available but very limited study is reported from North India. We have made an attempt to study the symptomatic children using various simple cost-effective methods. Male preponderance was higher than females (55.3% against 44.7%) which are similar to other studies from India and abroad.[9] Of the total cases 45.1% cases were in the group A (infants to 1 years) followed by that in group B (29.2%). The results obtained was near to a study reported from Bangalore where 40.2% cases were less than 1 year, 33.4% were in the age group of 1 to 5 years and 18.4% were more than 5 years. Clinical manifestations are usually severe and are often lethal during infancy and early part of life.[10]

There is a significant association of different age groups with developmental delay (p=0.004). Others studies reported that seizure, lethargy, delayed milestone, poor feeding and recurrent vomiting are the common features of IEM.[11] Symptoms occur mostly in 1 year of age and their diagnosis is based on clinical manifestation.[12]

In our study, familial characteristics revealed 27.6% consanguinity whereas sibling deaths due to unknown cause was 13.6% and only 5.7% had family history of inherited metabolic disorders. Due to consanguinity the rate of mortality and morbidity are generally increased. Consanguineous marriages are the main reason for such high prevalence in Muslim majority countries like in Saudi Arabia.[13] Similarly, the frequency of consanguineous marriages was prominently higher among children of Pakistani, Afgan, Turkish and other Arab countries including Egypt , Iraq, Jordan, and Tunisia.[14-16] In our country, South India has higher rate of consanguinity as

Table 2: Association of familial characteristics with region

S. No.	Familial characteristics	Region		P value
		Rural N (%)	Urban N (%)	
1.	Consanguinity (73)	60 (82.2)	13 (17.8)	0.09
2.	Sibling illness/deaths (36)	27 (75.0)	9 (25.0)	1.00
3.	Family history (15)	13 (86.7)	2 (13.3)	0.28

Table 3: Association of consanguinity with biochemical parameters

S.No.	Biochemical parameters	Consanguineous Mean±SD	Non consanguineous Mean±SD	P value
1.	pH	7.38±0.05	7.37±0.07	0.35
2.	Urea (mg/dL)	32.90±15.07	34.81±28.34	0.58
3.	Serum Creatinine(mg/dL)	0.64±0.27	0.67±0.36	0.59
4.	Total Protein (g/dL)	5.94±1.31	6.01±1.36	0.70
5.	Albumin (g/dL)	2.99±0.78	3.14±0.93	0.24
6.	Total Bilirubin (mg/dL)	0.94±0.93	0.89±0.86	0.67
7.	Direct Bilirubin (mg/dL)	0.39±0.36	0.33±0.31	0.21
8.	Hemoglobin (g/dL)	9.46±2.05	10.24±2.27	**0.01***
10.	Na^+ (mEq/L)	137.50±5.72	137.84±5.58	0.66
11.	K^+ (mEq/L)	4.02±0.83	3.98±0.70	0.68
12.	HCO_3^- (mEq/L)	20.96±2.50	20.66±2.82	0.42
13.	Blood sugar (mg/dL)	83.30±19.51	84.50±13.23	0.56
14.	Alkaline phosphatase (U/L)	344.43±178.00	362.71±212.00	0.51
15.	ALT (U/L)	36.00±24.33	33.47±21.09	0.40
16.	AST (U/L)	38.04±19.24	38.70±23.51	0.92

HCO_3^- bicarbonate, ALT – alanine transaminase, AST – aspartate transaminase, Na^+ - sodium, K^+ - potassium, * significant at p<0.05

Table 4: Age wise distribution of clinical presentation

S. No.	Clinical Presentation	Infant -1 years (N=119) (%) Group A	1.1– 5 years (N=77) (%) Group B	5.1 - 15 years (N=68) (%) Group C	P value
1.	H/o Lethargy (59)	27 (22.7)	15 (19.5)	17 (25.0)	0.72
2.	Recurrent vomiting (46)	22 (18.5)	13 (16.8)	11 (16.2)	0.91
3.	Developmental delay (47)	12 (10.1)	22 (28.5)	13 (19.1)	**0.004***
4.	Convulsion (33)	15 (12.6)	9 (11.7)	9 (13.2)	0.96
5.	Jaundice (29)	18 (15.1)	5 (6.5)	6 (7.7)	0.13
6.	Mental retardation (20)	6 (5.0)	7 (9.1)	7 (10.3)	0.35
7.	Hypoglycemia (27)	18 (15.1)	5 (6.5)	4 (5.9)	0.06
8.	Skeletal deformities (8)	3 (2.5)	2 (2.6)	3 (4.4)	0.74
9.	Coarse facial (8)	4 (3.3)	1 (1.3)	3 (4.4))	0.52
10.	Microcephaly (7)	2 (1.7)	1 (1.3)	4 (5.9)	0.15
11.	Acidosis (39)	19 (15.9)	8 (11.1)	12 (17.6)	0.41

* statistically significant, p<0.05

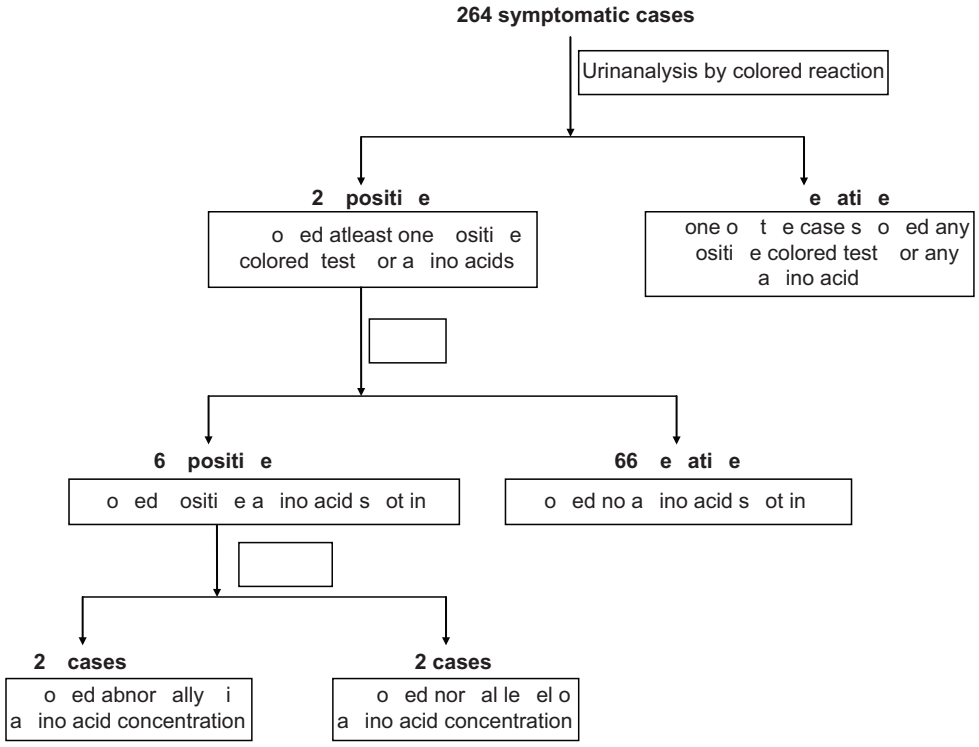

Figure 1: Flow chart of distribution of cases

Table 5: Age wise analysis of biochemical parameters

SS.	Biochemical parameters	Infant to 1 year (Mean±SD) Group A	1.1 to 5 years (Mean±SD) Group B	5.1 to 15 years (Mean±SD) Group C	P value
1.	pH	7.37±0.07	7.38±0.05	7.38±0.08	0.40
2.	Blood Urea(mg/dL)	35.06±22.75	29.86±14.75	37.92±36.46	0.14
3.	Serum Creatinine(mg/dL)	0.70±0.38	0.58±0.22	0.70±0.37	**0.04***
4.	Total Protein (g/dL)	5.54±1.37	6.05±1.14	6.73±1.18	**<0.01***
5.	Albumin (g/dL)	2.89±0.89	3.08±0.86	3.49±0.82	**<0.01***
6.	Total Bilirubin (mg/dL)	1.10±1.09	0.71±0.65	0.74±0.57	**<0.01***
7.	Direct Bilirubin (mg/dL)	0.41±0.38	0.29±0.27	0.31±0.25	**0.02***
8.	Hemoglobin (g/dL)	9.96±2.45	10.13±2.06	10.00±2.04	0.86
10.	Na^+ (mEq/L)	137.61±16.86	138.17±4.50	137.51±4.15	0.73
11.	K^+ (mEq/L)	3.99±0.83	4.07±0.68	3.91±0.61	0.43
12.	HCO_3^- (mEq/L)	20.77±2.80	20.62±2.75	20.83±2.63	0.89
13.	Blood sugar (mg/dL)	81.42±16.41	85.60±13.54	87.35±14.09	0.02*
14.	Alkaline phosphatase (U/L)	364.40±233.46	348.19±173.32	356.57±177.52	0.86
15.	Serum ALT (U/L)	40.12±27.33	28.10±16.22	30.64±13.10	<0.01*
16.	Serum AST (U/L)	43.59±25.60	34.20±17.24	34.92±19.84	<0.01*

HCO_3^- bicarbonate, ALT – alanine transaminase, AST – aspartate transaminase, Na^+ - sodium,K^+ - potassium, * significant at p<0.05

Table 6: Screening of urine samples by color reactions

S.No.	Color Tests in urine samples	Infant to 1 year positive cases (Group A)	1.1–5 years positive cases (Group B)	5.1–15 years positive cases (Group C)	Total Positive (%)
1.	Rothera's test	24	16	12	52 (19.7)
2.	Ferric chloride test	1	3	2	6 (2.3)
3.	DNPH test	5	0	2	7 (2.7)
4.	Nitrosonaphthol test	4	2	1	7 (2.7)
5.	Homogentisic acid test for alkaptonuria	0	1	1	2 (0.8)
6.	Benedict's test	15	11	7	33 (12.5)
7.	Seliwanoff's test	7	3	1	11 (4.2)

Table 7: TLC of amino acids in urine samples in different age groups

Elevated amino acids/reducing sugars	Infant to 1 year (Group A)	1.1–5 years (Group B)	5.1–15 years (Group C)	No. of positives cases (%)
Phenylalanine	2	1	0	3 (2.4)
Tyrosine	6	2	2	10 (7.9)
Branched chain amino acids	7	4	2	13 (10.2)
Glycine	6	2	1	9 (7.1)
Generalised aminoaciduria	12	4	10	26 (20.5)
Total No.	33	13	15	61

Table 8: Elevated amino acids level using HPLC method in different age groups

Elevated amino acids	Infant to 1 year (Group A)	1.1–5 years (Group B)	5.1–15 years (Group C)	No. of positives cases (%)
Phenylalanine	1	0	0	1 (3.5)
Tyrosine	3	2	0	5 (17.2)
MSUD	4	1	1	6 (20.7)
Glycine	4	0	1	5 (17.2)
Generalised aminoaciduria	7	2	3	12 (41.4)
Total	19	5	5	29

compared to North India.[17] Consanguinity rate was also higher among the people belonging to rural areas (82.2%) and frequently low in urban region (17.8%). The increased rate of consanguineous marriages has been noticed to be associated with illiteracy, low socioeconomic status, and rural residence.[13,18]

In this study mean hemoglobin level was lower than the normal reference range. The incidence of anemia is high in developing countries whereas in poor countries it is significantly high due to nutrients deficiency.[19] Anemia was a common feature in group A of our study which is similar to the findings of Rivera and Amor (77.2%) (2003) and Sanabria et al. (2000) who have reported moderate to severe anemia prevalence in 43.4% and 5.8% respectively of their studied cases (2000). Similarly in India the prevalence of anemia was 72.9% in a study conducted at Bangalore[20] and 56% in Mangalore.[21] In IEM, jaundice or liver dysfunctions are progressive and usually appear at the end of the first or during the second week of life with vomiting, diarrhea, poor weight gain, and eventually cataract formation.[22]

From our study, based on the two screening tests, i.e. color reactions and TLC, it was found that generalised aminoaciduria as the common amino acid disorder followed by MSUD and tyrosinuria. Patil (2009) reported generalized aminoaciduria as the most common amino acid disorder in their study group as well.[9] Generalized aminoaciduria was found the most prominent abnormality, comprising 70% by paper chromatography.[23] Rao et. al. (1991) had used TLC in a screening program and reported tyrosinemia, MSUD, glycinemia and PKU as common aminoaciduria in their study.[10]

In our study, further analysis of TLC positive cases by HPLC revealed overall 10.9% cases with elevated amino acids in symptomatic children. Among the common aminoacidopathies found in our study was generalized aminoaciduria, MSUD followed by tyrosinuria, glycinuria and phenylketonuria. Similar findings have also been reported from a study in North India by Kaur et al.[24] They have reported homocystinuria, alkaptonuria, MSUD and nonketotic hyperglycinaemia as the most common aminoacidopathies. Contrary to this study and our study, Jailkhani et. al. (2008) has found phenylketonuria as most common findings other than branched chain amino acids disorder.[11] In our study, among the 6 cases (2.2%) confirmed for MSUD by three screening tests, 4 of them were below the age of 5 years. All these 6 cases were showing elevated amino acids in their plasma samples as well. A large study conducted in Malaysia showed less than 1% MSUD in Malay children suspected for IEM.[25] In this study, out of the 29 cases of aminoacidopathies, tyrosinuria was positive in 5 cases (17.2%) and overall 1.9% among all symptomatic cases. Out of this 60% (3/5) were in the early age, i.e, below 1 year. Glycinuria was also detected in my study. Studies from other parts of India have found glycinuria to be among the commonest IEM associated with mental retardation. It is common for patients with any of the IEM to indicate either one or more dysmorphic features or abnormality that are nonspecific. This disorder is caused by defects in enzyme in the different components of the mitochondrial glycine cleavage enzyme system and is presented soon after birth with lethargy, hypotonia, myoclonus, and apnea.[26] There was only case positive for phenylketonuria after analyzing through 3 screening tests. It is common in Western countries leading to mental retardation. In India it is reported to be between 1.13% to 1.2% mentally retarded children.[17,24] Phenylalanine builds up in blood

and brain to non-conversion to tyrosine or it lacks the enzyme phenylalanine hydroxylase. This leads to high level of phenylalanine in brain, which in turn is responsible for inadequate neurotransmitter synthesis, intellectual disability and abnormal behavioral outcome.[27]

CONCLUSION

This study concludes that clinical evaluation is important for the definitive diagnosis of IEM, helping in the adequate managing of the investigation. Secondly, this study concludes the significant clinical burden and it emphasizes the role of consanguinity in increasing the predilection for these recessive disorders in countries with high consanguinity rate. The three screening tests i.e. color reactions, TLC and HPLC should to be carried out in the laboratory before reaching a confirmation using TMS. This will lessen the financial burden on parents as analysis by TMS or GC/MS is very expensive in our health care set up. Urinalysis presents cheap and, in some cases, allow us to suggest a more sensitive investigation. Hence the routine screening must not be overlooked especially in the background of high birth rate and preventable morbidities in our country. This study focused on increasing awareness of clinicians and pediatricians about the importance of suspecting the possibility of IEM in children and their early detection by using a set of rapid, easy and cost effective simple tests for screening, especially in symptomatic group.

ACKNOWLEDGEMENT

We are thankful to all enrolled patients in the study and their parents for their cooperation. We are also thankful to the laboratory technical staff of Departments of Biochemistry and Pediatrics, King George's Medical University, Lucknow, India, for the help and cooperation in carrying out this work.

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Author's Contribution:
GKS, ST, AAM- Conceptualized and designed the study; **NF, ST-** Screened the children based on inclusion and exclusion criteria; **NF, SS-** Did the laboratory investigations and experimental work; **NF**- Drafted the manuscript; **AAM, GKS-** Revised the manuscript before submission. All the authors have contributed substantially in manuscript preparation, read and approved the final manuscript.

Work attributed to:
Department of Biochemistry, King George's Medical University, Lucknow- 226003, India.

Orcid ID:
Dr. Nazmin Fatima - https://orcid.org/0000-0003-2040-6596
Dr. Gyanendra Kumar Sonkar - https://orcid.org/0000-0002-0813-5466
Dr. Shalini Tripathi - https://orcid.org/0000-0003-0805-7370
Dr. Sangeeta Singh - https://orcid.org/0000-0003-0189-8759
Dr. Abbas Ali Mahdi - https://orcid.org/0000-0002-8580-9911

Source of Funding: Research work was funded by Council of Science and Technology, Uttar Pradesh (CST-UP), India, (Grant No. CST/YSS/D-2874),
Conflict of Interest: None.