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ORIGINAL ARTICLE

Selective Screening for Organic Acidurias and Amino Acidopathies in Pakistani Children

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Abstract

Objective: To determine the frequency of organic acidurais (OA) and amino acidopathies (AA) in selected high-risk patients screened in two years.

Study Design: Retrospective Observational study.

Place and Duration of Study: The Aga Khan University Hospital (AKUH), Karachi, from January 2013 to December 2014.

Methodology: Patients with OA and AA were included in the study and patients with IMDs other than OA and AA were excluded. Amino acids and organic acids were analyzed on high performance liquid chromatography and gas chromatography-mass spectrometry respectively. Clinical data and chromatograms of patients screened for IMDs were reviewed by chemical pathologist and metabolic physician.

Results: Eighty-eight cases (4.7%) were diagnosed including 41 OA (46.5%), 28 AA (31.8%) and 19 others (21.5%) from 1,866 specimens analyzed. Median age of the patients was 1.1 years, with high consanguinity rate (64.8%). Among OA, methyl CoA mutase deficiency was diagnosed in 9 (10.2%) and was suspected in 2 (2.3%) cases. Five (5.7%) cases of MHBD (2-methyl-3-hydroxybutyryl-CoA), 4 (4.5%) each of PPA (propionic aciduria) and HMG-CoA lyase deficiency, 3 (3.4%) cases each of IVA (isovaleric aciduria), multiple carboxylase deficiency, fructose-1, 6-biphosphatase deficiency, fumarase deficiency, GA-1 (glutaric aciduria type 1) and 2 (2.3%) cases of EMA (ethyl-malonic aciduria). AA included 8 (9.1%) cases of MSUD (maple syrup urine disease), 6 (6.8%) cases of CBS (cystathionine beta-synthetase) and UCDs (urea cycle disorders) each, 5 (5.7%) cases of hyperphenylalaninemia and 3 (3.4%) cases of hyperprolinemia were reported. Other inherited metabolic disorders included: 9 (10.2%) cases of intracellular cobalamin defects, 2 (2.3%) cases each of alkaptonuria, Canavan's disease, SUCL (succinate CoA ligase) deficiency, and 1 (1.1%) case each of DPD (dihydropyrimidine) deficiency, GA-2, NKH (non-ketotic hyperglycinemia), AADC (aromatic amino acid decarboxylase) deficiency.

Conclusion: This study presents frequency of OA and AA in the high-risk Pakistani pediatric population analyzed locally.

Key Words: *Inherited metabolic disorders. Organic acidurias. Amino acidopathies. HPLC. GCMS. Pakistan.*

INTRODUCTION

Amino acidopathies (AA) and organic acidurias (OA) are single gene inherited metabolic disorders (IMDs) due to absolute or relative deficiency of specific enzyme or co-enzyme.[1] This leads to accumulation of toxic metabolites, which manifest as clinical and biochemical abnormalities. They are mostly inherited as autosomal recessive (AR) trait.[2] The IMDs are rare diseases with individual incidence of less than 1 per 100,000 births; but as a group, the incidence may approach 1 in 800 to 2500 births.[3,4]

Hutchesson *et al*. reported 100 times higher incidence of tyrosinaemia type I and non-ketotic hyperglycinemia (NKH) in Pakistani population as compared to the North-West Europeans in UK. Authors reported a collective frequency of 1:318 for IMDs in Pakistani population in West Midlands, UK.[5]

Pakistan has a high infant mortality rate of 57.48 deaths/1,000 live births and ranks third highest among Asian countries. It is also world's seventh most-populous country with an estimated population of over 196,174,380 in 2015, with 33% of the population below the age of 15 years. Sixty percent of all marriages are consanguineous.[6] The burden of IMDs is expected to be high in Pakistan due to high consanguinity rate but exact data is lacking. Newborn screening for any IMD does not exist at national level. The healthcare system is mainly hospital-based with ineffective primary health care facilities. Most deliveries are attended by traditional birth attendants at home.

Diagnosis of IMDs in Pakistan has been challenging due to resource constraints and limited clinical and technical expertise. From 2008-12, all diagnostic tests were out-sourced, as there was no local IMDs diagnostic facility. During this period, 85 patients were diagnosed.[7] Later, the local diagnostic Biochemical Genetics Laboratory (BGL) was established at The Aga Khan University Hospital (AKUH), Karachi. The burden of IMD in this part of the world is not known and the aim of this study was to determine the frequency of various OA and AA in high-risk patients.

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METHODOLOGY

This retrospective observational study was conducted at BGL in the Section of Clinical Chemistry, Department of Pathology and Laboratory Medicine, AKUH-K from 2013-14. All patients with high clinical suspicion of OA and AA were included in the study on samples received from all over Pakistan through its 200 Phlebotomy Centers. All patients with IMDs other than OA and AA, like glycogen storage disorders, lysosomal storage disorders, peroxisomal disorders, and mitochondrial disorders were excluded from the study. A retrospective review of AA and OA data was done. The study was exempted from ethical approval by the Ethical Review Committee of the Hospital (3654-Path-ERC-15). For amino acid chromatography, 3-4 ml of blood samples were collected in lithium heparin tube or 0.5 ml CSF in sterile container; and for organic acid analysis, 2-5 ml urine was collected in preservative-free container. Samples were sent to BGL after centrifugation from outreach phlebotomy centers and transported in dry ice, according to the standard protocol. All samples were stored at -20°C prior to analysis. A questionnaire comprising of clinical and biochemical information was filled for AKUH-K in-patient and out-patient. Similar questionnaire was filled for samples received at phlebotomy centers of AKUH spread across Pakistan.

The amino acids quantification tests were performed by Cation-Exchange HPLC on Biochrom 30+ model 440/570 nm, using lithium column diameter 4.6 mm. Standard, control and specimens (plasma and urine) were deproteinized with sulfosalicylic acid (10%) and nor leucine was added as internal standard, whereas deproteinization was not performed for CSF specimens.

The column eluent was mixed with ninhydrin reagent, forming colored compound and the amount of color produced was directly proportional to the quantity of amino acids present in the sample. Each colored compound was determined at 440 nm for hydroxypoline and proline, and rest of the AAs were estimated at 570 nm. The results were analyzed by EZ chrome software version 3.31.[8]

Urine organic acids samples were analyzed by Gas Chromatography Mass Spectrometry (GCMS) (Model 7890 / 5975, Agilent Technologies Santa Clara, CA). Estimation of urine creatinine (mmol/l) was done in all the samples. Organic acids were extracted from specimens by liquid-liquid extraction after mixing the internal standard solution (3, 3 dimethyl glutaric acid), followed by oximation, extraction and derivatization.[8,9] Eluent was analyzed with mass detection on scan mode data acquisition, whereas quantification of succinylacetone was done on SIM mode data acquisition. The data generated from mass detector was analyzed by Chem station software with the help of libraries, like ORGASID, ACID97 and NIST.

Quality control, validation and proficiency testing validation for amino acids and organic acids were performed, according to Clinical and Laboratory Standards Institution guidelines at optimization of assays. Precision, accuracy, carry over, analytical measurement range/ linearity and lot to lot verification were validated. We used commercially available quality controls (ClinChek 1 and 2 and SKML) and ran two levels of controls with every batch of 10 samples. BGL also participate in proficiency testing surveys from European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism (ERNDIM) and College of American Pathologists (CAP) of plasma amino acids, urine organic acids and succinylacetone, since its inception.

AA were diagnosed on the pattern of abnormalities resulting from deranged quantification of amino acids specific to disorders.[10] Diagnoses of OA were confirmed by disease specific urinary metabolites on chromatogram.[11] One thousand, eight hundred and sixty-six specimens were analyzed at BGL during 2013-14. Out of this, 88 positive cases of OA and AA were reviewed by Chemical Pathologist and Metabolic Physician before releasing final result.

Statistical Package for Social Sciences version 21 was used to analyze the data. Frequencies and percentages were calculated for gender, consanguinity, sibling deaths, geographical distribution of IMDs, and clinical presentations. Different OA, AA and other IMDs were identified and frequencies and percentages were analyzed for each specific IMD. Data of age distribution was skewed so median value was reported along with interquartile ranges.

RESULTS

Eighty-eight cases (4.7%) were diagnosed with IMDs from 1866 specimens analyzed over the 2 years period from all over the country. The geographical distribution of IMD cases was 59 from Sindh (67%), 22 from Punjab (25%), 04 from Khyber Pakhtunkhwa (4.5%), and 03 from Baluchistan (3.4%).

Median age was 1.1 years (IQR 2-4 years). More than half were males 50 (56.8%). About two-thirds (64.8%) of the patients were born to consanguineous parents. Most frequent clinical presentations were lethargy (n=39, 44%), developmental delays (34, 39%), poor feeding (n=34, 39%) and vomiting (n=28, 32%). Other presentations were hypotonia (n=24, 28%), mental retardation and fever (n=21, 24% each), and seizures and failure to thrive (n=13, 15% each). Most of the patients had combination of the mentioned symptoms and signs. Developmental delays were more frequently noted in infants, whereas metabolic decompensation was more frequent in 1-5 years age group.

Forty-one patients (47%), were reported with OA. Twenty-eight patients (32%) were reported to have amino acidopathies and are summarized in Table I. Nineteen other IMDs (21.6%) identified included: intracellular cobalamin defects, alkaptonuria, canavan's

disease, succinate CoA ligase (SUCL) deficiency, dihydropyrimidine (DPD) deficiency, aromatic amino acid decarboxylase (AADC) deficiency, glutaric aciduria type 2 (GA-2), and non-ketotic hyperglycinemia (NKH).

DISCUSSION

This is a novel data from Pakistan, which shows frequency of OA, AA and other IMDs established through local diagnostic facility. Testings for OA, AA and other IMDs in previously published five studies were sent abroad for diagnostic purposes.[7,12-15] This is cumbersome as critical time is lost in procuring the results, which delays the timely acute treatment; thus results in either morbidity or mortality.

Table I: Frequency of IMDs reported from Biochemical Genetics Laboratory, AKUH (2013-2014).

Organic Aciduria: n = 41 (47%)	
Methyl CoA mutase deficiency	9 (10.2%)
MHBD	5 (5.7%)
HMG CoA lyase deficiency	4 (4.5%)
Propionic aciduria	4 (4.5%)
Isovaleric aciduria	3 (3.4%)
Multiple carboxylase deficiency	3 (3.4%)
Fructose-1, 6-biphosphatase deficiency	3 (3.4%)
Fumarase deficiency	3 (3.4%)
Glutaric aciduria type 1	3 (3.4%)
Suspected MMA	2 (2.3%)
Ethylmalonic aciduria	2 (2.3%)
Amino Acidopathies: n = 28 (32%)	
MSUD	8 (9.1%)
CBS deficiency	6 (6.8%)
Urea cycle disorders	6 (6.8%)
Hyperphenylal aninemia	5 (5.7%)
Hyperprolinemia	3 (3.4%)
Others: n = 19 (21.6%)	
Intracellular cobalamin defects	9 (10.2%)
Alkaptonuria	2 (2.3%)
Canavan's disease	2 (2.3%)
SUCL deficiency	2 (2.3%)
DPD deficiency	1 (1.1%)
AADC deficiency	1 (1.1%)
Glutaric aciduria type 2	1 (1.1%)
NKH	1 (1.1%)

MHBD = 2-methyl-3-hydroxybutyryl-CoA; HMG CoA lyase = 3-hydroxy-3-methylglutaryl-CoA; MSUD = maple syrup urine disease; CBS = cystathionine beta synthetase; SUCL = succinate CoA ligase; DPD = dihydropyrimidine; AADC = aromatic amino acid decarboxylase; NKH = Non-ketotic hyperglycinemia.

These studies showed that mostly the patient presented with acute metabolic decompensation and neurological manifestations. Three studies reported increased frequency of OA, UCDs and NKH in Pakistani population.[12-14] According to Cheema *et al*.[15], the most frequent metabolic disorders were glycogen storage disorders, gaucher disease, galactosemia and MPS as compared to other IMDs. This major difference in frequency could be due to the fact that this study was conducted at the gastroenterology and hepatology department, where mostly the patient present with liver manifestations.

Afroze *et al*. reported 85 patients with IMD in 5 years period from 2008-2012 diagnosed at AKUH-K, of which 47 were OA and AA. During that period, all diagnostic tests were outsourced to IMR, Malaysia.[7] After the local availability of the diagnostic tests for the OA and AA, the number of diagnosis made in 2 years period is almost double the number of OA and AA that were diagnosed in 5 years period when only the clinical service was available. This highlights the fact that the easy accessibility of a local diagnostic facility to physicians and patients leads to more patients being diagnosed and eventually results in timely management of patients with IMDs.

In this study of high-risk patients, it was found that the most common OA in the population was MMA, followed by MHBD, PPA and HMG CoA lyase deficiency. In AA; MSUD, CBS deficiency and UCDs were most frequent. Hori *et al*. from Japan analyzed 4,653 patients and reported 79 (70%) cases of OA and 21 (19%) cases of AA, performed on GC-MS. The most common disorder was MMA, followed by PPA, OTCD, and multiple carboxylase deficiency.[16] This data is similar to the frequencies presently identified in the local population.

Tan *et al*. from Singapore analyzed 3,656 patients and reported 41 (32%) cases of OA, performed on GC-MS; and 48 (37%) cases with AA, performed on amino acid analyzer.The most common OAs were GA-2, PPA, and MMA.[17] In their study, AAs disorders identified were OTCD, phenylketonuria, MSUD and NKH. Thong *et al*. from Malaysia analyzed 13,500 patients and reported 98 (37%) cases with OA, performed on GC-MS; and 132 (50%) cases with AA, performed through the ion-exchange HPLC.[8]

Table II: Inherited metabolic disorders data from local studies.

Year	Author	Study Subjects	Population	Common Presentation	IEM Testing Facility	Common Inherited Disorders
1994	P. Multi *et al.*[12]	21 NICU patients with hyperammonemia	Karachi	Neurological manifestation (76 %)	India, UK	UCDs, OA, MSUD, IVA and NKH
2008-2012	Afroze *et al.*[7]	426 patients	Karachi	Psychomotor retardation (36%) Seizures (23.7%)	Kuala Lumpur, Malaysia.	OA, PAA, Lysosomal storage disorders, glycogen storage disorders
2009	H. satwani *et al.*[13]	62 outpatient children from Pediatric clinic	Karachi	Developmental delays (47.6%) and respiratory distress (39.3%)	Japan	OA and UCDs
2013	S. Choudary *et al.*[14]	10 neonates	Islamabad	Seizures (50%) and Coma (50%)	India	MMA, NKH, Frutose-1, 6-bisphosphatase deficiency and Biotinidase deficiency
2011-14	Cheema HA *et al.* [15]	239 Children <14 years	Lahore	Metabolic crisis (32.2%), liver failure (15.5%) and mental retardation (13.3%)	Country not mentioned only stated "diagnostic testing for metabolic disorders were sent abroad"	Glycogen storage disorders, Gaucher disease, galactosemia and MPS

Overall, the frequency of OA as compared to AA is higher in this study (47% vs. 33%) which is similar to Japanese population (70% vs. 19%); whereas Malaysia,[8] Singapore[17] and Thailand[18] showed increased frequency of AA as compared to OA, i.e. 50% vs. 37%, 37% vs. 32%, 55% vs. 33%, respectively. When compared to the incidence of IMDs with other Asian countries, Pakistan has higher disease prevalence of IMDs (4.7%) as compared to Singapore (3.5%), Japan (2.4%), and Malaysia (2%). This variation in detection rates of IMDs could be due to difference in the sample size and consanguinity rate in these countries.[16-18]

For better outcome for treatable IMDs, early and correct diagnosis is essential.[19] Confirmatory testing, enzyme assays, and/or molecular characterization after suggestive biochemical diagnosis, will help in prenatal genetic counselling and prenatal genetic diagnosis for the couple at risk. Carrier screening for at risk family members followed by genetic counselling for married couples and pre-marital genetic counselling for carrier to make informed decisions to choose their partners will help in reducing the overall burden of AR IMDs in Pakistan. These diagnostic modalities involve heavy investment, expertise and robust healthcare to support the service.[20] Apart from this, high-risk screening for IMDs provides important insight into the disease pattern of a country, which can provide critical information required for the selection of the most prevalent IMDs, to be included in the national newborn screening for IMDs. Thus, efforts to establish more diagnostic facilities and allocation of national funds and resources to address the common IMDs can be a way forward in strengthening the IMD services and hope for patients and families fighting with IMDs in Pakistan.

CONCLUSION

This study presents frequency of OA and AA in the high-risk Pakistani pediatric population through selective high-risk screening in symptomatic patients. Local availability to the diagnosis of OA and AA increases the access to patients and physicians, leading to timely diagnosis and saving critical time to initiate timely disease-specific treatment, leading to eventual better management and effective genetic counselling. This locally generated data will be helpful in planning and providing services for patients with IMDs, including newborn screening.

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