Inborn errors of metabolism screening and profiling of amino acids, acylcarnitines and glucose-6-phosphate dehydrogenase deficiency in Bangladesh population

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Certificate

This is to certify that the thesis entitled "**Inborn errors of metabolism screening and profiling of amino acids, acylcarnitines and glucose-6-phosphate dehydrogenase deficiency in Bangladesh population**" has been carried out by Suprovath Kumar Sarker (Registration No: 93, Session: 2015-2016) under our supervision. This is further to certify that it is an original work and suitable for partial fulfillment of the degree of doctor of philosophy in Genetic Engineering and Biotechnology, University of Dhaka.

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Dedications

To

My Parents

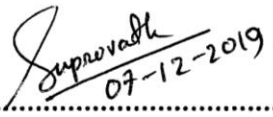
Sushanta Kumar Sarker and Archana Rani Sarker

&

My Country, Bangladesh

DECLARATION

I hereby declare that this thesis entitled "**Inborn errors of metabolism screening and profiling of amino acids, acylcarnitines and glucose-6-phosphate dehydrogenase deficiency in Bangladesh population**" is an original work and contains no material which has been submitted elsewhere for any other degree or diploma in any university or equivalent institution. To the best of my knowledge, the thesis contains no material which has been published previously or written by another person, except where due references have been provided in the text of the thesis.



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Abstract

Similar to other lower and lower middle income countries, Bangladesh is facing the accelerated demographic shift from communicable diseases to non-communicable diseases (NCDs) including genetic disorders (GDs). Approximately 10,000 single-gene disorders have been identified which affects millions of people worldwide. One of the major categories of phenotypically and genotypically heterogeneous group of genetic disorders are inborn errors of metabolism (IEM). Liquid Chromatography tandem mass spectrometry (LC-MS/MS) is commonly used for the diagnosis of more than 30 IEM. Accurate and reliable diagnosis of IEM by quantifying amino acids (AAs) and acylcarnitines (ACs) using LC-MS/MS systems depend on the establishment of age-specific cut-off values of the analytes for the population under investigation. Thus, the first objective of the study was to (1) determine the age-specific cut-off values of AAs and ACs in Bangladesh and (2) validate the LC-MS/MS method for diagnosis of the patients with IEM. A total of 570 healthy participants were enrolled in this study. They were divided into 3 age groups, namely, (1) newborns (1-7 days), (2) 8 days–7 years, and (3) 8–17 years, to establish the age-specific cut-offs for AAs and ACs. In addition, 273 suspected patients with IEM were enrolled to evaluate the reliability of the established cut-off values. Among these patients, nine cases came out as screening positive by LC-MS/MS and seven cases were confirmed by urinary GC-MS analysis including 3 cases with phenylketonuria, 1 with methylmalonic acidemia, 1 with isovaleric acidemia, 1 with citrullinemia type II, and 1 with carnitine uptake defect. Two borderline positive cases with medium-chain acyl-CoA dehydrogenase deficiency were negative by urinary GC-MS analysis.

The second objective of the study was to detect mutations in glucose-6-phosphate dehydrogenase (G6PD) gene that are responsible for G6PD deficiency in Bangladeshi individuals because G6PD deficiency is also very common like IEM in the Indian subcontinent. Among 121 clinically suspected patients, 12 (11 males and one female) patients were found to be G6PD deficient, suggesting the frequency of G6PD deficiency among suspected patients is 9.9%. Sanger sequencing revealed c.C131G substitution (exon-3: Ala44Gly) in six samples, c.G487A substitution (exon 6: Gly163Ser) in five samples and c.G949A substitution (exon-9: Glu317Lys) in the coding sequence in one sample. From the study, it appears that Ala44Gly and Gly163Ser are the most common G6PD mutations in Bangladesh.

The third objective of the study was to establish a real-time PCR-based approach called high-resolution melt (HRM) analysis as a rapid and reliable method for the detection of G6PD variants in heterozygous females. Although hemizygous males and homozygous females are easily detected by the conventional G6PD enzyme assay method, the heterozygous state can be missed by the conventional methods as the mosaic population of both normal and deficient RBCs circulate in the blood. Sixty-three clinically suspected females were evaluated for G6PD status using both enzyme assay and HRM analysis. Four out of sixty-three participants came out as G6PD deficient by the enzyme assay method, whereas the HRM approach could identify nine participants with different G6PD variants, namely one homozygous and eight heterozygous.

In conclusion, along with the establishment of a validated LC-MS/MS method for the quantitation of amino acids and acylcarnitines from the DBS cards, the study also demonstrates the presence of predominant IEM in Bangladesh. In addition, this study is the first report for the underlying genetic defects of the G6PD gene that causes G6PD deficiency in the Bangladeshi population and the establishment of a rapid and reliable HRM-based method for identification of G6PD variants among heterozygous females in Bangladesh.

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List of abbreviations

5´UTR	5´ untranslated region
AAs	Amino acids
ACMG	American College of Medical Genetics
ACs	Acylcarnitines
BIRDEM	Bangladesh Institute of Research & Rehabilitation in Diabetes, Endocrine and Metabolic Disorders
BLAST	Basic Local Alignment Search Tool
BMRC	Bangladesh Medical Research Council
BSMMU	Bangladesh, Bangabandhu Sheikh Mujib Medical University
CACT	Carnitine-acylcarnitine translocase deficiency
CAH	congenital adrenal hyperplasia
CF	cystic fibrosis
CIT	Citrullinemia
CPS	counted per second
CPT I	Carnitine palmitoyltransferase I deficiency
CPT II	Carnitine palmitoyltransferase II deficiency
CTD	Carnitine transporter deficiency)
CUD	Carnitine uptake deficiency
CV	coefficient of variance
DBS	Dried blood spot
dsDNA	double-stranded DNA
DSH	Bangladesh and Dhaka Shishu Hospital
EDTA	Ethylenediaminetetraacetate
ESI	Electron spray ionization
FIA-ESI-MS/MS	Flow injection analysis-electrospray ionization-tandem mass spectrometry
FST	fluorescent spot test
G6PD	Glucose-6-phosphate dehydrogenase
GA-I	Glutaric aciduria type 1
GALT	Galactosemia
GC-MS	Gas chromatography-mass spectrometry and
GDs	Genetic disorders
GEB	Genetic Engineering and Biotechnology
HCY	Homocystinuria
HHH	hyperornithinemia-hyperammonemia-homocitrullinemia
HPLC	High-performance liquid chromatography

HRM	High resolution melt
ideSHi	Institute for Developing Science and Health Initiatives
IEM	Inborn errors of metabolism
IVA	Isovaleric acidemia
LCHAD	Long-chain 3-hydroxyacyl-CoA dehydrogenase
LC-MS/MS	Liquid Chromatography tandem mass spectrometry
MCAD	Medium-chain acyl-CoA dehydrogenase deficiency
MDG-4	Millennium Development Goals 4
MMA	Methylmalonic acidemia
MRM	Multiple reaction monitoring
MSUD	Maple syrup urine disease
NBS	Newborn screening
NCDs	Non-communicable diseases
NINS	National Institute of Neurosciences & Hospital
PA	propionic aciduria
PKU	Phenylketonuria
QC	quality control
RE	relative error
SCAD	Short-chain acyl-CoA dehydrogenase deficiency deficiency
SCHAD	Short-chain 3-OH acyl-CoA dehydrogenase
SD	standard deviations
SDGs	Sustainable development goals
TFP	Trifunctional protein deficiency
TMS	Tandem mass spectrometry
U5MR	Under-five mortality rate
VLCAD	Very long-chain acyl-CoA dehydrogenase deficiency

INTRODUCTION

CHAPTER 1

1. Introduction

1.1. Background

Bangladesh has achieved remarkable success in fulfilling Millennium Development Goals 4 (MDG-4) targeting child survival over the last couple of decades and the under-five mortality rate (U5MR) in Bangladesh has declined significantly from 144 to 32 per 1000 live births between 1990 and 2017 [1]. Much of this improvement can be attributed to successful immunization coverage, improving universal breastfeeding practices, control of communicable diseases and vitamin supplementation. However, there are other barriers to further reduce child deaths, morbidity, and disabilities in life. In this respect, especially non-communicable diseases (NCDs) are huge barriers to lowering child mortality and morbidity in Bangladesh. NCDs have been recognized as a major challenge for sustainable development goals (SDGs) at the United Nations Summit on Sustainable Development in September 2015 [1, 2]. To achieve the SDG goal by 2030, premature deaths from NCDs have to be reduced by one-third [2]. To achieve this goal, the specific control strategy for NCDs including genetic disorders (GDs) should be taken, especially for the disorders with high prevalence in Bangladesh. Although, in countries like Bangladesh data about the percentage of GDs are limited due to lack of epidemiological studies and accurate diagnostic facility, it is expected that the prevalence of GDs such as inborn errors of metabolism (IEM) and glucose-6-phosphate dehydrogenase (G6PD) deficiency is common in Bangladeshi population because the prevalence of these disorders are very high in India. The prevalence of IEM in India is about 1 in 1000 live births and G6PD deficiency is 1 in 10 for male individuals [3, 4]. As the geographical and ethnic background of India and Bangladesh is similar, it can be considered that the prevalence of these disorders will be similar or even slightly higher in our country as consanguinity among the Bangladeshi population is high, compared to India where it is prohibited in some regions. In Bangladesh as well as other developing countries, we are still lagging behind in the use of diagnostic testing for detecting infants and children with these disorders. For this reason, GD suspected patients' specimens are being sent to other developed countries for differential diagnosis of IEM. However, analyzing specimens in other countries is unaffordable for most Bangladeshis. Thus, the development and strengthening of our facilities for genetic testing of GDs such as IEM and G6PD deficiency as well as the establishment of a cost-effective and reliable diagnostic method for these disorders will be greatly beneficial for our country.

1.2. Inborn errors of metabolism (IEM)

IEM are phenotypically and genotypically heterogeneous group of disorders caused by defective and altered activity of the necessary enzymes or proteins [5]. Due to defective metabolic pathways, substrates of the specific metabolic pathways are either accumulated or products behind the defective enzymes remain deficient [6]. Individually these disorders are very rare, most having an incidence of less than 1 per 100,000 births but collectively they are numerous [7, 8]. When considered collectively, the incidence is about 1 in 800 to 1 in 2500 births [5, 9-11]. However, the incidence of IEM may be underestimated because of the lack of awareness and diagnostic errors. Etiologies of these diseases are often multifactorial and 40% of these cases are idiopathic but the prevalence rate is high in populations with consanguineous marriages [12]. Remarkably, one-third of the IEM are characterized by involvement of the nervous system and the repercussions of neurometabolic changes, particularly to neurological development during the early years of life make it imperative to detect and treat them at the earliest to prevent forthcoming disaster. In case of availability of treatment, the quality of life of patients with IEM can be made better by early diagnosis and treatment initiation.

1.2.1. Major categories of inborn errors of metabolism (IEM)

Based on the affected metabolic pathway, the IEM disorders are broadly classified as disorders of amino acid metabolism, organic acid metabolism, fatty acid oxidation, carbohydrate metabolism or lysosomal storage disorders. In recent years, large numbers of IEM have been discovered which do not fall into the above-mentioned broad categories. A list of major categories of IEM with the most common examples of IEM have given in Table 1.1.

Table 1.1: Major categories of IEM with the most common examples of IEM.

Categories of IEM	Examples
Disorders of amino acid metabolism (aminoacidopathies)	Phenylketonuria, homocystinuria, glutaric acidemia type 1
Disorders of organic acid metabolism (organic acidurias)	Methylmalonic aciduria, isovaleric acidemia, alkaptonuria, 2-hydroxyglutaric acidurias, propionic acidemia, maple syrup urine disease
Disorders of fatty acid oxidation	Carnitine uptake deficiency, carnitine palmitoyltransferase 1 deficiency, medium-chain acyl-CoA dehydrogenase deficiency, long-chain hydroxyacyl-CoA dehydrogenase deficiency

Table 1.1 (continued).

Urea cycle disorders	Citrullinemia, argininemia, ornithine transcarbamylase deficiency, argininosuccinic aciduria
Lysosomal storage disorders	Niemann–Pick disease, Gaucher's disease
Carbohydrate disorders	Fructose-1,6-diphosphatase deficiency, hereditary fructose intolerance, galactosemia, glycogen storage diseases
Mitochondrial disorders	Pyruvate carboxylase deficiency, Phosphoenolpyruvate carboxykinase deficiency, Pyruvate dehydrogenase complex deficiency, Oxidative-phosphorylation (respiratory chain) defects

1.2.2. Prevalence of IEM in different countries

Most incidence rates of IEM have been revealed from the screening of high-risk patients or expanded newborn screening (NBS) programs in different countries [5]. Different research groups from India have reported that IEM are not very uncommon among the Indian population. A study conducted by Devi *et al.* (among 18,300 newborns) revealed that the overall prevalence rate of IEM is 1 in 1000 live births in India [3]. Another multi-center (Mumbai, Delhi, and Baroda) study revealed that the most common aminoacidopathies in India are tyrosinemia, maple syrup urine disease, and phenylketonuria. According to this study, the overall incidence rate of metabolic disorder is 1 in 2497 (among 112,269 newborns) [13].

Table 1.2: Incidence rate of common metabolic disorders in India [3].

Metabolic Disorder	Screened	Confirmed	Incidence
Amino Acid	18,300	5	1:3600
Phenylketonuria	18,300	1	1:18300
Transient Tyrosinemia	18,300	2	1:9000
Hyperglycinemia	18,300	1	1:18300
Hyperargininemia	18,300	1	1:18300
Galactosemia (GALT)	10,300	1	1:10300
G6PD	8,800	4	1:2200

Study conducted by Applegarth *et al.* in British Columbia, Canada during 1968 to 1996, reported that the incidence of IEM was 1 in 2500 [9]. Sanderson *et al.* reported from the United Kingdom that the incidence rate of IEM is 1 in 784 [10]. A study conducted by Moammar *et al.* in Saudi Arabia demonstrated that the incidence rate of IEM was 1 in 667 [14]. The rate of IEM in Hong Kong was determined by Lee *et al.* in 2011 and the study revealed that the rate was 1 in 4122 [15]. According to a national retrospective survey in Italy by Dionisi-Vici *et al.* demonstrated that the incidence rate of IEM was 1 in 3707 (Table 1.3) [16].

The data from the expanded newborn screening (NBS) programs in different countries have reported the incidence of amino acid disorders, organic acidemias, and fatty acid oxidation disorders which has been shown in Table 1.3. The incidence rate of IEM varies among different countries because different panels of IEM disorders were screened in different countries [5]. Moreover, the prevalence of different IEM varies depending on the ethnicity of the population [17].

Table 1.3: Incidence of IEM in different countries.

Country	Incidence rate of IEM (among live births)	Reference
Canada	1 in 2500	Applegarth *et al.* [9]
Italy	1 in 3707	Dionisi-Vici *et al.* [16]
United Kingdom	1 in 784	Sanderson *et al.* [10]
Saudi Arabia	1 in 667	Moammar *et al.* [14]
Hong Kong	1 in 4122	Lee *et al.* [15]
United States	1 in 3810	Zytkovicz *et al.* [18]
Mainland China	1 in 5800	Gu *et al.* [19]
Japan	1 in 9330	Yamaguchi *et al.* [20]
Australia	1 in 6000	Wilcken *et al.* [21]
Korea	1 in 2000	Yoon *et al.* [22]
India	1 in 3600	Devi *et al.* [3]
Taiwan	1 in 5882	Niu *et al.* [23]
Germany	1 in 2400	Schulze *et al.* [24]

1.2.3. Signs and symptoms of IEM

According to the type of IEM and the severity of disease-causing mutations, the clinical manifestations of IEM patients vary. In the case of severe mutations, clinical manifestations are presented within the first few days of life or neonatal period, whereas in the case of mild mutations the symptoms are not presented until childhood or adulthood. Moreover, the clinical manifestations of the same disorder may vary in a child than an adult. Even the different individuals with the same disorder manifest different symptoms. Although clinical manifestations of IEM are variable, major clinical features in an infant with metabolic disorders include:

- Lethargy
- Poor feeding
- Decreased body temperature
- Vomiting
- Tachypnea (related to acidosis)
- Decreased perfusion
- Abnormal muscular rigidity
- Seizure

In addition, most children with IEM have an elevated lactate level, hyperammonemia, metabolic acidosis, hypoglycemia, or elevated level of ketone or reducing sugar in the urine.

If the patients with IEM are not treated, these symptoms may progress rapidly to coma, organ failure, respiratory arrest or even death. It is important to mention here that the set of symptoms are disease-specific and not every IEM patient will present all of the symptoms. In addition to the above-mentioned symptoms, the patients with IEM may manifest following clinical features:

- Developmental delay
- Failure to thrive
- Recurrent vomiting, diarrhea, abdominal pain
- Problems with nerve pain
- Movement disorders
- Muscle cramps
- Hearing or visual problems
- Organomegaly such as lymphadenopathy and hepatosplenomegaly
- Jaundice and liver problems
- Unusual odor
- Behavioral and psychiatric problems
- Dysmorphic features
- Skin rash or abnormal pigmentation
- Heart problems, like cardiomyopathy or heart attack

1.2.4 Techniques used for IEM screening

To date, about 1000 IEM disorders have been identified [5]. Testing for each of these disorders individually is burdensome and practically impossible. With the development of liquid chromatography-mass spectroscopy (LC-MS/MS) also known as tandem mass spectrometry (TMS), it has become possible to simultaneously detect the amount of many metabolites in a single sample. IEM has been implemented in various countries including developed and developing countries through expanded newborn screening programs for most common IEM. Dried blood spots are used as a primary sample for IEM screening. Abnormalities identified in the dried blood spot analysis may need to be confirmed with other tests such as various biochemical tests, gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) or the involvement of enzymatic assay and/or DNA based molecular analysis.

1.2.5 IEM screening panel in a different country

LC-MS/MS is used for the screening of aminoacidopathies, organic acidemia, and fatty acid oxidation disorders in the majority of the countries. This test panel includes semi-quantitative measurement of alanine (Ala), citrulline (Cit), leucine including isoleucine (leu and Ile), glycine (Gly) , methionine (Met), phenylalanine (Phe), valine (Val), ornithine (Orn), proline (Pro), tyrosine (Tyr), free carnitine (C0), acetylcarnitine (C2), propionylcarnitine (C3), butyrylcarnitine (C4), isovalerylcarnitine (C5) , glutarylcarnitine (C5-DC) , hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), dodecanoylcarnitine (C12), tetradecanoylcarnitine (C14), hexadecanoylcarnitine (C16), and octadecanoylcarnitine (C18) metabolites to detect more than 30 IEM from dried blood spot (DBS) (Table 1.4) [24].

Table 1.4: List of inborn errors of metabolism (IEM) detected by MS/MS analysis [24].

Amino acidemias	Organic acidemias	Fatty acid oxidation disorders
Phenylketonuria (PKU)	propionic aciduria (PA)	Short-chain acyl-CoA dehydrogenase deficiency (SCAD) deficiency
Tyrosinemia type I (TYR-I)	Methylmalonic acidemia (MMA)	Short-chain 3-OH acyl-CoA dehydrogenase (SCHAD) deficiency
Non-ketotic hyperglycinemia (NKH)	Cobalamin disorders A, B, C, D, F (cbl)	Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency
Maple syrup urine disease (MSUD)	Isovaleric acidemia (IVA)	Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency
Citrullinemia (CIT)	Glutaric aciduria type 1 (GA-I)	Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency
Argininosuccinate lyase (ASL)	3-methylcrotonyl-CoA carboxylase deficiency (3-MCC)	Trifunctional protein deficiency (TFP)
Homocystinuria (HCY)	Holocarboxylase synthetase deficiency	Carnitine transporter deficiency (CTD) or carnitine uptake deficiency (CUD)
Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH) Syndrome	Biotinidase deficiency	Carnitine palmitoyltransferase I (CPT I) deficiency
Arginase deficiency	HMG-CoA lyase deficiency	Carnitine palmitoyltransferase II (CPT II) deficiency

Table 1.4 (continued).

Arginase deficiency	HMG-CoA lyase deficiency	Carnitine-acylcarnitine translocase (CACT) deficiency
Hyperornithinemia	Methylglutaconic aciduria	
Hyperprolinemia	Multiple acyl-CoA dehydrogenase (MAD, GAII) deficiency	
Hyperhydroxyprolinemia	Isobutyryl-CoA dehydrogenase deficiency	
Glycine N-methyltransferase deficiency	2-methylbutyryl-CoA dehydrogenase deficiency	
Hypermethioninemia other than HCY	Ethylmalonic encephalopathy	
	Medium-chain 3-ketoacyl-coa thiolase deficiency	
	Succinyl CoA transferase (SCOT) deficiency	
	Beta-ketothiolase (BKT) deficiency	
	Malonic aciduria	

The panel screened by Newborn Screening (NBS) varies from one country to another. Table 1.5 summarizes the IEM test panel for NBS in different countries. In the USA, 19 IEM including LCHADD, CPT-II, VLCADD, SCADD, multiple acyl-CoA dehydrogenase deficiency, long-chain acyl-CoA dehydrogenase deficiency, propionic acidemia, methylmalonic acidemia, IVA, glutaric aciduria type I, beta-ketothiolase deficiency, beta-methylcrotonyl-CoA carboxylase deficiency, HMG-CoA lyase deficiency, tyrosinemia types I and II, citrullinemia type I, argininemia, hyperornithinemia-hyperammonemia-homocitrullinemia (HHH) syndrome, and argininosuccinic acidemia were started at the University of Massachusetts Medical School. IEM are screened using TMS or LC-MS/MS

in other developed countries like Canada, Austria, England, France, Germany, Italy, Spain, Australia, and New Zealand (Table 1.5). India has started NBS programs in some states in recent years. Congenital hypothyroidism (CH), glucose-6-phosphate dehydrogenase (G6PD) deficiency, congenital adrenal hyperplasia (CAH), cystic fibrosis (CF) and galactosemia (GAL) are screened along with 45 IEM using LC-MS/MS (TMS) in India.

Table 1.5: Screening panel of Newborn Screening (NBS) in various countries.

Country/Territory	Conditions screened
North America [25]	
USA	At least 29 core conditions recommended by the American College of Medical Genetics (ACMG). Some states screened up to 70 conditions
Canada	CH, PKU, TMS (up to 38 conditions)
Europe [26]	
Austria	PKU, MSUD, Tyr-I, MCADD, LCHADD,VLCADD, CPI-I CPT-II, CACT, CTD, KTD, HMG, MMA, PA, IVA,GA-I, 3MCCC
England	PKU, MCADD, CF, CH, Sickle cell disease
France	PKU, MSUD, MCADD, LCHADD, VLCADD, CPI-I CPT-II, CACT, IVA, GA-I
Germany	PKU, MSUD, Tyr-I, Cit, ASL, HCY, MCADD, LCHADD, VLCADD, CPI-I CPT-II, CACT, CTD, KTD, HMG, MMA, PA, IVA, GA-I, 3MCCC
Italy	PKU, MSUD, HCY, Tyr-I, MCADD, LCHADD, VLCADD, HMG, IVA, GA-I, 3MCCC
Spain	PKU, MCADD
Australasia [27]	
Australia	CH, PKU, GAL, MSUD, HCY, CF, TMS
New Zealand	CH, CAH, PKU, GAL, MSUD, HCY, CF, TMS
Asia [27, 28]	
Bangladesh	CH, PKU
Mainland China	CH, PKU, TMS (3–28 conditions)
Hong Kong SAR	CH, G6PD

Table 1.5 (continued).

India	CH, G6PD, CAH, CF, GAL, TMS (45 conditions)
Indonesia	CH
Laos	CH
Malaysia	CH, G6PD
Mongolia	CH, CAH
Pakistan	CH
Palau	CH, CAH, PKU, GAL
Philippines	CH, CAH, PKU, GAL, G6PD
Sri Lanka	CH
South Korea	CH, PKU, (optional for GAL, MSUD, HCY, HIS)
Taiwan	CH, HCY, PKU, GAL, G6PD, CAH, TMS
Thailand	CH, PKU, TMS
Vietnam	CH, CAH, G6PD

Abbreviations used only in this table: ASL: argininosuccinate lyase deficiency; CACT: carnitine acylcarnitine translocase deficiency; CAH: congenital adrenal hyperplasia; CF: cystic fibrosis; CH: congenital hypothyroidism; Cit: hypercitrullinemia; CTD: carnitine transporter defect; GAL: galactosemia; GA-I: glutaric aciduria type I; HCY: homocystinuria; HIS: histinidinemia; HMG: HMG-CoA lyase deficiency; KTD: beta ketothiolase deficiency; MMA methylmalonic acidemia; PA: propionic acidemia; TMS: TMS-based screening for various conditions; Tyr-I: tyrosinemia type I. Congenital deafness and other non-IEMs conditions were not included in this table.

Although screening of congenital hypothyroidism among newborns was started in a public hospital facility in Bangladesh, the screening program has been discontinued. Moreover, the number of newborns screened under this program was not significant compared to the annual birth cohort of Bangladesh. Currently, there is no national newborn screening program for IEM in Bangladesh.

1.2.6 Importance of establishment of IEM screening facility in Bangladesh

According to the WHO, the birth cohort of Bangladesh is more than 3 million and by considering that 0.1% (1 in 1000 live births) newborns affected by IEM in Bangladesh, it can be estimated that more than 3000 newborns are affected by these disorders each year. Moreover, most of these disorders remain undetected during the first weeks of life and if these disorders are not treated in the very early life of the patients, the patients will suffer from irreversible mental damage, which not only causes economic burden to the affected patients' family but also to the health care settings of the country. For many of the IEM

diseases, specific and effective treatments are available and the worsening of the disease can be prevented by early therapeutic interventions [10]. Even if therapy is unavailable, an accurate diagnosis is crucial for genetic counseling to prevent future births in families [29].

Many of the IEM can be detected early in life using mass spectrometry-based analysis and initiation of early treatment can save the valuable life and mental retardation of the affected child can be prevented. Some of the classical IEM though have very specific symptoms, they often overlap with symptoms of sepsis and perinatal events [30-32]. As the facilities for genetic testing of these disorders are limited, specimens are sent to other developed countries for diagnosis which is costly, time-consuming and results are often unreliable. For this reason, the development of our own facility for reliable diagnosis of IEM will be greatly beneficial to the families, infants and children. This will help in the rapid detection of the defects and early treatment to minimize irreparable damage to newborns and suspected patients with IEM.

1.3. Glucose-6-phosphate dehydrogenase deficiency

Along with the IEM panel, Glucose-6-phosphate dehydrogenase (G6PD) deficiency is screened through the NBS program in different Asian countries such as India, Taiwan, Hong Kong, Malaysia, Philippines and Vietnam (Table 1.5) [5] and G6PD deficiency is one of the most common X-linked recessive genetic disorder in Indian subcontinent [3, 33]. Although in most cases, G6PD-deficient individuals appear normal, it can lead to life-threatening anemia under conditions of oxidative stress induced by foods (fava beans, legumes), drugs (primaquine, sulfa drugs) and infection with microorganisms [34]. Table 1.6 shows the list of potential drugs and chemicals, which cause hemolysis in G6PD-deficient patients [35]. Additionally, some studies suggest that G6PD deficiency increases the risk of severe neonatal hyperbilirubinemia, which can lead to lifetime disability with kernicterus if inadequately treated [36-38]. Sometimes G6PD deficiency can be beneficial. For example, some studies have reported that G6PD deficiency provides resistance against malaria as the malaria parasite cannot complete its life cycle in compromised G6PD-deficient red blood cells (RBCs) with decreased life span [39, 40].

Table 1.6: Potential drugs and chemicals that cause hemolysis in patients with G6PD deficiency [35].

Drug Type	Definite association	Possible association	Doubtful association
Antimalarials	Primaquine Pamaquine	Chloroquine	Mepacrine Quinine
Sulfonamides	Sulfanilamide Sulfacetamide Sulfapyridine Sulfamethoxazole	Sulfadimidine Sulfasalazine Glibenclamide	Aldesulfone Sulfadiazine Sulfafurazole
Sulfones	Dapsone		
Nitrofurantoin	Nitrofurantoin		
Antipyretic or analgesic	Acetanilide	Aspirin	Paracetamol Phenacetin
Other drugs	Nalidixic acid Niridazole Methylthionium Phenazopyridine Co-trimoxazole	Ciprofl oxacin Chloramphenicol Vitamin K analogues Ascorbic acid Mesalazine	Aminosalicylic acid Doxorubicin Probenecid Dimercaprol
Other chemicals	Naphthalene 2,4,6-trinitrotoluene	Acalypha indica extract	

1.3.1. Prevalence of G6PD deficiency in a different country

Glucose-6-phosphate dehydrogenase deficiency is the most common inherited enzyme deficiency and about 400 million people are affected by this disorder worldwide [41]. Globally, the prevalence rate of G6PD deficiency is 4.9% [37, 42, 43] and the prevalence in malaria-endemic countries is higher (8%) [44]. The average prevalence rate of G6PD has been illustrated in Fig 1.1. The average prevalence of G6PD in the Indian subcontinent including Bangladesh is between 8% to 13% [41]. According to WHO, the prevalence of G6PD deficiency is 0-10% in India and it is higher among the tribal population [45]. The prevalence of G6PD varies from 2.3% to 27.0% in different ethnic groups in India and the overall rate of G6PD deficiency is 7.7% [46, 47]. Mehmood *et al.* reported that the prevalence of G6PD deficiency is 4.5% in Pakistan [48]. Data from several locally

published journals showed that the incidence rate of G6PD deficiency among Pakistani male is 2% to 4% [49]. A study conducted by Li *et al.* demonstrated that the prevalence of G6PD deficiency among the Kachin (Jingpo) ethnic group of China-Myanmar border is 29.6% [50]. On the other hand, Gunawardena *et al.* reported from Sri Lanka that 13.95% and 7.97% were G6PD deficient at Anuradhapura and Kurunegala districts, respectively [51]. Ghimire *et al.* showed that the prevalence rate of G6PD deficiency varied between 2.83% to 9.82% in different regions of Nepal [52]. These data indicated that G6PD deficiency is very common in the Indian subcontinent.

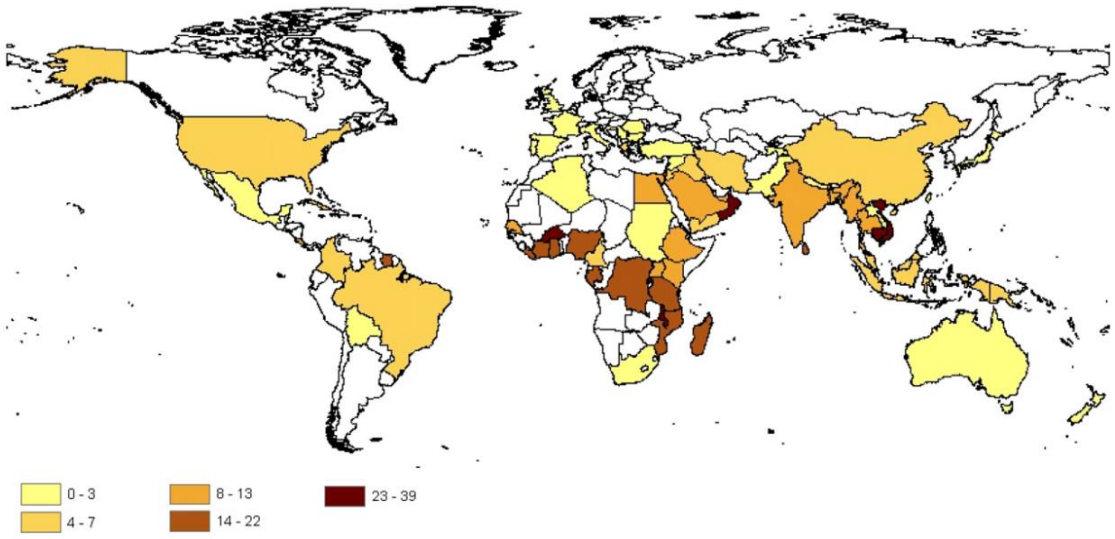


Fig 1.1: Average G6PD prevalence (%) in different countries *[41].*

1.3.2. Gene involved in G6PD deficiency and structure of G6PD gene

Glucose-6-phosphate dehydrogenase gene is located on a cytogenetical position Xq28 of long (q) arm of the X chromosome (Fig 1.2). The molecular location of the G6PD gene on the X chromosome is from the base pairs 154,531,390 to 154,547,586 (Homo sapiens Annotation Release 109, GRCh38.p12). The sequence of 20,114 bp of human DNA was sequenced by Chen *et al.* in 1991 including the G6PD gene [53]. The human G6PD gene has 13 exons which span 18kb of the X chromosome [54] and the protein-coding region of the G6PD gene is divided into 12 segments and the size of the segments is 12 to 236 bp [54]. The G6PD protein consists of 531 amino acids and the molecular mass of the protein is 58 kD [55].

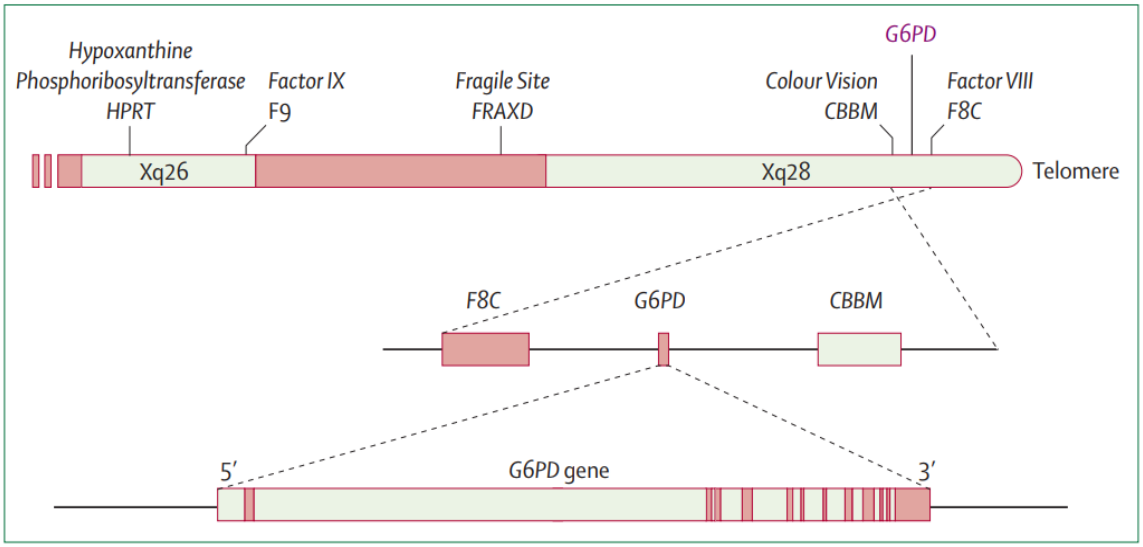


Fig 1.2: Chromosomal location of glucose-6-phosphate dehydrogenase (G6PD). The telomeric region of the X chromosome harbors the G6PD gene [35].

1.3.3. G6PD gene variants responsible for G6PD deficiency

Based on different electrophoretic and biochemical characteristics, more than 400 variants of G6PD enzymes have been identified [35]. Although all variants of G6PD do not show reduced enzyme activity, numerous point mutations in the G6PD gene are responsible for enzyme instability which causes G6PD deficiency in erythrocytes [56]. More than 200 mutations have been identified worldwide. G6PD mutation database was updated by Minucci *et al.* in 2012 and it showed that out of 186 mutations in the G6PD gene, 159 (85.4%) had single-nucleotide substitutions, 10 (5.3%) had deletions and 15 (8.0%) are multiple mutations with two or more substitutions, 10 (5.3%) are deletions, and 2 (1.0%) are mutations that affect introns [57].

The most common variant of G6PD in Greece, Italy, Spain, the Middle East and the Indian subcontinent and parts of South-East Asia is the Mediterranean variant [58]. The mutations A376G and G202A are present in the G6PD A– allele which is the commonest G6PD deficiency allele in Africa and America (Fig 1.3). Viangchan, Mahidol, Mediterranean, and Canton (G1376T) are the commonest variants in South-East Asian countries [58]. Kaeda *et al.* reported that G6PD Mediterranean, G6PD Orissa, and G6PD Kerala-Kalyan were the most common mutations in India [59]. Jamornthanyawat *et al.* reported that the Mediterranean variant of G6PD was the most common variant among many ethnic groups in Afghanistan [60]. Moiz *et al.* reported from Pakistan that the G6PD Mediterranean variant was present in Southern Pakistan [61]. G6PD Orissa and G6PD Mediterranean variants are very common in Maharashtra, Odisha, and Gujarat [47, 62-64]. In addition,

G6PD Chatam, G6PD Coimbra, G6PD Nilgiri, and G6PD Gond had also been reported among different tribal groups in India [63, 65, 66]. G6PD Namoru (208 T→C) variant was found in the southern Indian region including Nilgiri district and Tamil Nadu [67]. The G6PD variants in different regions of the world have been shown in Fig 1.3 [58, 68].

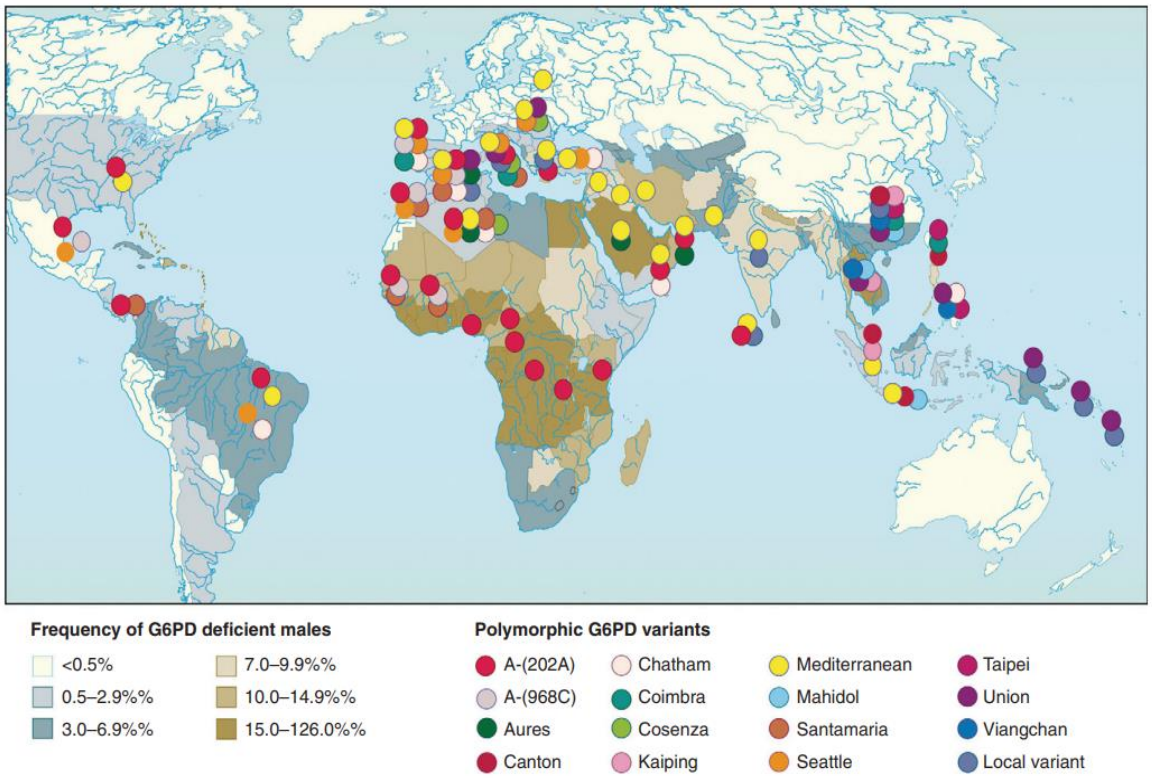


Fig 1.3: World map showing glucose-6-phosphate dehydrogenase (G6PD) variants in different countries. The shadings indicate the overall prevalence of G6PD deficiency in individual countries, whereas the distribution of individual G6PD variants is shown by colored symbols [58, 68].

1.3.4 Classification of G6PD deficiency according to the WHO

According to the WHO, genetic variants of G6PD are divided into five classes based on G6PD enzyme activity levels and among the first three classes are considered as deficient states (Table 1.7) [58, 69, 70]. G6PD enzyme activity levels of Class I, Class II, Class III, Class IV, and Class V G6PD-deficient patients are < 1%, 1–10%, 10-60%, 60-150% and > 150% of normal G6PD activity, respectively. Class II deficient patients are more common in Asian and Mediterranean populations and Class III deficient patients are found among 10% of black males in the United States (Table 1.7).

Table 1.7: WHO classification of glucose-6-phosphate dehydrogenase deficiency by severity [58, 69].

Class	Severity of G6PD deficiency	Percentage of normal G6PD activity	Prevalence
I	Very severe, associated with chronic non-spherocytic hemolytic anemia	< 1	Uncommon occur across populations
II	Severe	1 – 10	Varies; more common in Asian and Mediterranean populations
III	Moderate	10 – 60	10% of black males in the United States
IV	Normal activity	60 – 150	Rare
V	Increased activity	> 150	Rare

1.3.5 Techniques used for G6PD deficiency diagnosis

The fluorescent spot test (FST) and quantitative G6PD enzyme assay methods are based on the principle of measurement of NADPH produced from $NADP^+$ by the G6PD enzyme. Another G6PD screening test is done using the decolorization of brilliant cresyl blue dye. Although qualitative tests for G6PD can be used for the detection of G6PD deficiency in males and homozygous females, heterozygous females cannot be detected using a qualitative G6PD test.

A recently developed kit for G6PD deficiency detection is BinaxNOW® (Abbott Laboratories, Illinois, USA). Although this qualitative G6PD deficiency detection kit can detect samples with enzyme activity level less than 4.0 U/g Hb with high sensitivity (98%) and specificity (97–98%), this kit is not suitable for most tropical environments because this test must be performed at 18-25˚C [71]. Moreover, the test cost per sample is US$ 25, which is also very high for low-income countries. Another commercial kit for the qualitative detection of G6PD is CareStart™ (ACCESS BIO INC., Gyeongsangbuk-do, Korea) but the sensitivity of the kit is very low (68%). Another point-of-care device for

qualitative measurement of G6PD enzyme activity is CareStart™ G6PD biosensor system (ACCESS BIO INC., Gyeongsangbuk-do, Korea). However, the reliability and performance of the device are yet to be tested [58].

1.3.6 Importance of genetic analysis of G6PD deficiency and development of HRM-based methodology development

G6PD deficiency is the most common monogenic genetic disorder in South Asia but there is no data available about the prevalence of G6PD deficiency in Bangladesh. Moreover, there no molecular biological research has been conducted to reveal the G6PD gene mutations, which are responsible for this deficiency in the Bangladeshi population. For this reason, epidemiological and molecular biological research is essential for G6PD deficiency among the Bangladeshi individuals, which can be used for planning future research and the establishment of novel DNA-based diagnosis methods such as HRM-based method for this disorder in Bangladesh.

1.4. Rationality and the expected outcome of the study

The diagnosis of IEM involves the measurement of specific metabolites in either whole blood or plasma. Further workup includes metabolic analysis of the urine. The normal ranges of metabolites within a population depend both on the method and machine that are used to analyze the patients' samples as well as the population being measured. In the diagnosis of metabolic disorders, accuracy is of extreme importance. A false-negative result might cause a patient to suffer preventable illness or death. Although false positives are unavoidable given the rarity of individual metabolic disorders. A high false-positive rate adds a burden to the system in performing confirmatory tests and might give undue anxiety to a patient or family member. The key to minimizing both false negatives and false positives is the selection of a sensitive and specific cut-off value for the metabolites based on the method, machine, and population being tested. As we establish testing of IEM in Bangladesh, we must first establish the normal ranges of metabolites in our population using the method and machine in laboratory facilities. The reference range must be established for various age groups because the metabolic profile changes with the changes in diet and behavior as a child grow.

In addition to the IEM, molecular analysis for the G6PD gene to determine the mutations, which are responsible for G6PD deficiency in the Bangladeshi population, is extremely useful as this GD is very common in this region. Molecular analysis of the G6PD gene will

be useful to design the DNA-based method for genotyping of G6PD variants. Typical screening tests using G6PD enzyme assay may be reliably good to detect hemizygous G6PD deficient males and homozygous G6PD deficient females, these approaches may fail to detect heterozygous females. Screening for risk assessment should be done to exclude the G6PD-deficient individuals before the administration of an antimalarial drug such as primaquine. Heterozygous females with a higher proportion of normal to deficient RBCs population in the circulation are often misdiagnosed as normal in these screening tests [72, 73]. Thus, DNA-based high throughput, rapid and reliable mutation screening approaches such as high-resolution melting (HRM) curve analysis can effectively separate wild-type status from hemizygous, homozygous and heterozygous statuses [74].

In this study, a validated LC-MS/MS-based method for the quantification of amino acids and acylcarnitines, and reference values of these metabolites for three different age groups of our population has been established. Moreover, the investigation has been done to evaluate the reliability of the method and reference intervals by the screening of clinical specimens whether the established reference values of amino acids and acylcarnitines could be used to identify the IEM, especially aminoacidopathies, organic acidemias, and FAO disorders, among the clinically suspected patients with IEM in Bangladesh. In addition, this study is the first report of G6PD gene mutations, which are responsible for G6PD deficiency in the Bangladeshi population. The key finding of this study used to establish a cost-effective, reliable screening method for G6PD deficiency among heterozygous females in Bangladesh. The study is expected to motivate other researchers and Bangladeshi health policymakers to take necessary steps to establish genetic disorder research and diagnostic facility, which will ultimately reduce child mortality and morbidity in Bangladesh.

1.5 Objectives of the study

a. General objectives

Establishment of inborn errors of metabolism (IEM) and glucose-6-phosphate dehydrogenase (G6PD) deficiency screening protocol using high-throughput technologies like liquid chromatography-mass spectrometry (LC-MS/MS) and high-resolution melt (HRM) analysis in Bangladeshi participants.

b. Specific objectives

1. Setting up operational procedures of LC-MS/MS for quantitative analysis of amino acids and acylcarnitines using dried blood spot (DBS) cards for Bangladeshi participants.
2. Determination of age-dependent population reference values for amino acids and acylcarnitines from DBS using LC-MS/MS-based technology.
3. Determination of disease spectrum of aminoacidopathies, organic acidemias, and fatty acid oxidation disorders among clinically suspected patients with IEM in Bangladesh.
4. Determination of cut-off values for glucose-6-phosphate dehydrogenase enzyme activity in Bangladesh.
5. Investigate underlying genetic defects that are responsible for G6PD deficiency in Bangladeshi individuals.
6. Establishment of HRM-based high throughput screening method for the detection of G6PD variants in Bangladeshi individuals.

METHODS AND MATERIALS

CHAPTER 2

2. Methods and materials

2.1. Ethical approval and study population

2.1.1. Ethical approval

The study protocol for establishment of cut-off values of amino acids and acylcarnitines from dried blood spot (DBS) for diagnosis of inborn errors of metabolism (IEM) and molecular analysis of glucose-6-phosphate dehydrogenase gene mutations were reviewed and approved by the National Ethics Review Committee (NERC) of Bangladesh Medical Research Council (BMRC), Dhaka, Bangladesh (Ref: BMRC/NREC/2013-2016/990 and Ref: BMRC/NREC/2013-2016/107, respectively). In addition, this study was approved by the Ethical Review Committee of Faculty of Biological Sciences, University of Dhaka, Bangladesh (Date: 16.05.2017).

2.1.2. Study population and study sites

This study was conducted in the Department of Genetic Engineering and Biotechnology of the University of Dhaka (GEB), Bangladesh with the collaboration of Genetics and Genomics Laboratory of the Institute for developing Science and Health initiatives (ideSHi), Bangladesh.

2.1.3. Study participants and specimen collection sites for IEM study

A total of 570 healthy participants without any disease condition were enrolled for the study to establish the cut-off values of amino acids and acylcarnitines from DBS specimens using LC-MS/MS for the Bangladeshi population. The healthy participants were divided into three groups according to the age, namely, group A (age: 1-7 days), group B (age: 8 days-7 years) and group C (age: 8-17 years). The number of participants in group A, group B and group C were 120 (male: 54, female: 66), 243 (male: 124, female: 119) and 207 (male: 86, female: 121), respectively. The average (±SD) age of participants of group A, group B, and group C was 2.53±1.32 days, 4.56±1.89 years, and 11.83±2.70 years, respectively (Table 2.1).

Additionally, 273 suspected patients with signs and symptoms of IEM were also enrolled in the study to check the reliability of the method. Among these patients, 158 were males and 115 were females. The average (±SD) age of the suspected patients with IEM was 3.69±3.94 years (Table 2.1). Inclusion criteria for participation in the study were lethargy, irritation, poor feeding, tachypnea, seizures, persistent vomiting, toe-walking, unexplained

developmental delay, movement difficulty, speech problem, consanguinity of parents, positive family history with IEM including death of the previous sibling due to unexplained reason.

In addition, these patients were also subjected to investigation for signs such as metabolic acidosis with an increase in anion gap, persistent or recurrent hypoglycemia, hypotonia, hyperammonemia, splenomegaly, abnormal imaging and electrophysiologic findings, which are suggestive of metabolic disorders. However, the patients with the aforementioned symptoms who had a history of perinatal brain injury, infection of the central nervous system, or chromosomal abnormalities were excluded from the study. Written informed consent was obtained from the parents of suspected patients with IEM and healthy participants. The pediatric clinicians referred the suspected patients with IEM from the National Institute of Neurosciences & Hospital (NINS), Bangladesh, Bangabandhu Sheikh Mujib Medical University (BSMMU), Bangladesh and Dhaka Shishu Hospital (DSH), Bangladesh.

Table 2.1: Demographic characteristics of study participants.

Parameter	**Healthy control**			**IEM suspected Patient group (N = 273)**
	Group A (N = 120)	**Group B** (N = 243)	**Group C** (N=207)	
Average age[a]	2.53±1.32 days	4.56±1.89 years	11.83±2.70 years	3.69±3.94 years
Male[b]	54 (45%)	124 (51.0%)	86 (41.5%)	158 (57.9%)
Female[b]	66 (55%)	119 (49.0%)	121 (58.5%)	115 (42.1%)

[a]Values denote mean ± standard deviation. [b]Data presented as No. or N (%).

2.1.4. Study participants and specimen collection sites for molecular analysis of G6PD gene in Bangladeshi individuals

A total of 121 participants including 79 males and 42 females were enrolled for the molecular study of the G6PD gene in Bangladesh. The age range of the participants was 0–15 years and inclusion criteria for these participants were Bengali ethnicity, history of neonatal jaundice or a sudden onset of hemoglobinuria accompanied by pallor and hyperbilirubinemia. These study participants were enrolled from the out-patient departments of three hospitals, namely, Bangladesh Institute of Research & Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Dhaka, Bangladesh; Bangabandhu Sheikh Mujib Medical University (BSMMU); Dhaka, Bangladesh; Dhaka

Shishu Hospital (DSH); Dhaka, Bangladesh. Prior to enrollment for the study, we received the signed informed consent from the parents/legal guardians of the participants. Approximately, 2mL of venous blood was collected from each participant in an EDTA containing tube for fluorescent spot test (FST), G6PD enzyme assay, and Sanger's DNA sequencing for mutation analysis. For RNA extraction, another 1.0 mL blood without any anticoagulant was collected and aliquoted immediately in 4 microcentrifuge tubes (250 µL in each tube) containing 750 µL Trizol® LS (Life Technologies, USA).

2.1.5 Study participants and specimen collection sites for high-resolution melt curve (HRM) analysis of G6PD variants in heterozygous females

Based on family history or past clinical hemolytic complications, sixty-three female participants of Bangladeshi Bengali ethnic origin in the age range of 0–15 years were enrolled at the clinical settings of Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh; Bangladesh Institute of Research & Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM); and Dhaka Shishu Hospital (DSH), Dhaka, Bangladesh to establish the HRM-based method for analysis of G6PD variants in heterozygous females. The participants either belonged to a family with one or more G6PD deficient case(s) or had a history of recovery from an episode of neonatal hyperbilirubinemia or jaundice or hemoglobinuria accompanied by pallor or an accidental hemolytic crisis. It was made sure that the participants who had a history of the previous hemolytic crisis were in remission for a sufficient period of time so that the factors that have effect on G6PD enzyme activity level could be avoided, e.g., higher G6PD enzyme activity after recovery from a hemolytic crisis. One mL blood was collected by venipuncture from each of the participants in an EDTA-coated vacutainer. A fraction of the collected blood was used for G6PD enzyme assay, whereas another fraction was used for genomic DNA extraction.

2.2 Overview of the study design

There were three major objectives of the study:

Objective 1: Determination of age-dependent population reference values (cut-off values) for amino acids and acylcarnitines from DBS using LC-MS/MS-based technology for IEM screening in Bangladeshi individuals (denoted as "IEM screening study").

Objective 2: Investigate underlying genetic mutations that are responsible for G6PD deficiency in Bangladeshi individuals (denoted as "G6PD molecular study").

Objective 3: Establishment of HRM-based high throughput screening method for detection of G6PD variants in Bangladeshi participants (denoted as "G6PD-HRM study"). The overview of the study design has been illustrated in Fig 2.1(a), (b) and (c).

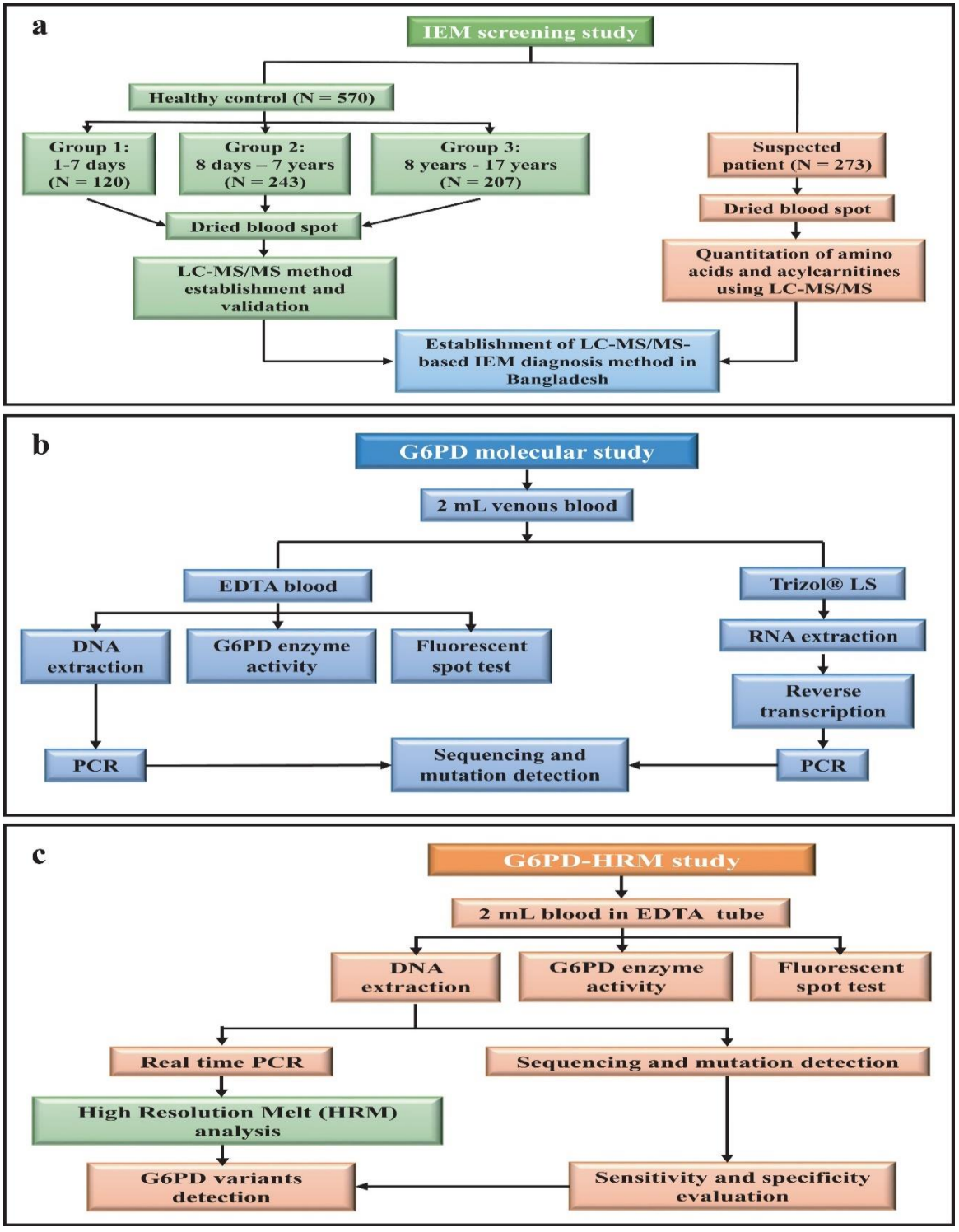


Fig 2.1: Overview of the study design. There were three major objectives of the study and presented as (a) IEM screening study, (b) G6PD molecular study, and (c) G6PD-HRM study. The flow diagram shows the overall study design and workflow of the study to achieve the study objectives.

2.3 Specimen collection and storage for IEM screening study

To prepare a dried blood spot (DBS) card for analysis by LC-MS/MS, whole blood was collected from newborns (age range: 24 hours – 7 days) by heel prick method and the blood spot was made by direct deposition on the Whatman™ 903 Generic Multipart filter paper (GE Healthcare, Westborough, MA, USA). Whole blood specimens for older children were collected after a 4-hour fasting period using standard venipuncture method and a DBS card was prepared by spotting 75 µL blood on Whatman™ 903 filter paper. For DNA isolation, the rest of the collected blood was transferred to a BD vacutainer containing di-potassium EDTA (Becton Dickinson, Franklin Lakes, NJ, USA) and stored at -70˚C freezer. The DBS cards were dried for 4 hours at room temperature and stored at -70˚C in a plastic ziplock bag with desiccants until analysis.

2.4 Quantitation of amino acids and acylcarnitines from dried blood spot using Liquid chromatography-mass spectrometry (LC-MS/MS)

LC-MS/MS analysis for the quantitation of amino acids and acylcarnitines was done using a NeoMass AAAC kit (Labsystems Diagnostics Oy, Finland). The specimen used for LC-MS/MS analysis was dried blood spot (DBS) cards, which were prepared using the blood of healthy controls and patients with suspected IEM. For the quantitation of these metabolites, the mass spectrometry-based technique was used where the compounds were ionized and fragmented into daughter ions using collision gas, which generates characteristic signature or fingerprint of each metabolite. This unique signature was used for the identification and quantification of each metabolite. Basic components of mass-spectrometers include the vacuum system, the ion source, the mass detector, and the computer for analysis. Electron spray ionization (ESI) technique was used for the ionization of the metabolites where ionization was done at atmospheric pressure. In the case of MS/MS or tandem mass spectrometry, two mass spectrometers work in a tandem manner to detect the precursor ions and daughter ions. In this method, amino acids and acylcarnitines from DBS were quantified without any chromatographic separation, which significantly reduces the analysis time.

2.4.1 Specimen preparation and LC-MS/MS analysis

Specimens were prepared for analysis using the NeoMass AAAC kit according to the manufacturer's instructions. Briefly, a vial of lyophilized isotope-labeled internal standards (IS) containing $^{2}H_4$-Alanine (Ala IS), $^{2}H_4$-^{13}C-Arginine (Arg IS), $^{2}H_2$- Citrulline (Cit IS),

$^{2}H_3$- Leucine (Leu IS), $^{13}C_6$-$^{15}N_2$- Lysine (Lys IS), $^{2}H_3$- Methionine (Met IS), $^{2}H_6$-Ornithine (Orn IS), $^{13}C_6$- Phenylalanine (Phe IS), $^{2}H_5$- Proline (Pro IS), $^{13}C_3$-Serine (Ser IS), $^{13}C_6$-Tyrosine (Tyr IS), $^{2}H_8$- Valine (Val IS), $^{2}H_9$- free carnitine (C0 IS), $^{2}H_3$- Acetylcarnitine (C2 IS), $^{2}H_3$- Propionylcarnitine (C3 IS), $^{2}H_3$- Butyrylcarnitine (C4 IS), $^{2}H_9$-Isovalerylcarnitine (C5 IS), $^{2}H_3$- Glutarylcarnitine (C5DC IS), $^{2}H_3$- Hexanoylcarnitine (C6 IS), $^{2}H_3$- Octanoylcarnitine (C8 IS), $^{2}H_3$- Decanoylcarnitine (C10 IS), $^{2}H_3$-Lauroylcarnitine (C12 IS), Myristoylcarnitine (C14 IS), $^{2}H_3$- Palmitoylcarnitine (C16 IS), and $^{2}H_3$- Stearoylcarnitin (C18 IS) was reconstituted by adding 1 mL of extraction solution which was provided with the NeoMass kit. To prepare the daily working extraction solution, the stock internal standard was reconstituted by diluting 1:100 (v/v) with the extraction solution provided with the kit. For analysis of amino acids and acylcarnitines, the stored DBS cards were brought to room temperature (+18 to +25˚C) prior to extraction and a 3.2 mm disk (equivalent to ~3.1 µL whole blood) was punched out using an automated Wallac Delfia DBS puncher (Perkin-Elmer Life Sciences, Inc.) into a well of polystyrene bottomed 96-well microplate provided with the kit. After the addition of 100 µL daily working extraction solution to each well of the microplate, the plate was covered with a piece of adhesive film. The plate was shaken at 650 rpm at room temperature using a microplate shaker. After shaking, 70 µL supernatant was transferred into a V-bottomed microplate and covered with aluminum foil to reduce the evaporation. LC-MS/MS analysis was done by placing the plate in the autosampler of the LC-MS/MS system and then injecting 5 µL supernatant into the LC-MS/MS system.

2.4.2 Instrumentation and LC-MS/MS analysis

Shimadzu LCMS-8050 liquid chromatograph-mass spectrometer (Shimadzu Corporation, Japan) equipped with a binary pump, autosampler, and electrospray ionization (ESI) source was used for the analysis. In addition, SCIEX 3200MD QTRAP LC-MS/MS system was used for this study (data not shown). Specimen analysis was done according to flow injection analysis-electrospray ionization-tandem mass spectrometry (FIA-ESI-MS/MS) method. For atmospheric pressure ionization of the metabolites, specimens were injected into the ESI source of LC-MS/MS system.

2.4.3 Data acquisition and data processing

The solvent delivery pump of the LC-MS/MS system was programmed to deliver the mobile phase (provided with the kit) at a constant flow rate of 150 µL/min and the multiple reaction monitoring (MRM) with positive ion mode was applied for metabolite detection

and quantitation. The ionization source parameters of LC-MS/MS included 250°C interface temperature, 400°C heat block temperature, 250°C desolvation line temperature, 4.5 kV interface voltage, 15.0 L/min drying gas flow, and 3.0 L/min nebulizing gas flow. Argon was used as collision gas at a pressure of 230 kPa. A Lab Solution (version 5.82 SP1, Shimadzu Corporation, Japan) software was used for data acquisition and data processing was done using a Neonatal Solution (version 2.20, Shimadzu Corporation, Japan). The total run time for each specimen was 1.5 minutes and the data were acquired for 0.9 minute. The data acquisition parameters of the MRM method for the analysis of amino acids and acylcarnitines have been shown in Table 2.2. The concentration of a target analyte was calculated by multiplying ion intensity ratio of the analyte and internal standard with the concentration of respective internal standards. A representative LC-MS/MS generated multiple reaction monitoring (MRM) chromatograms for deuterated internal standards has been shown in Fig 2.2.

To get the accurate test results, three different levels of quality control (QC) specimens, namely Low, Medium and High were provided with the NeoMass AAAC kit. The data generated from the LC-MS/MS analysis was monitored by extracting these QC specimens and analyzing them parallel with the specimens of healthy controls and patients.

Table 2.2: Data acquisition parameters of the MRM method for the analysis of amino acids and acylcarnitines.

Analytes	MRM (Analyte)	Internal Standard	MRM (Internal Standard)	Target Dwell Time (msec)	Target Q1 Pre Bias (V)	Target Collision Energy (V)	Target Q3 Pre Bias (V)
Ala	90.1→44.2	$^{2}H_4$-Ala	94.1→48.2	5	-11	-13	-19
Arg	175.1→70.1	$^{2}H_4$-^{13}C-Arg	180.1→75.1	15	-13	-24	-29
Cit	176.1→70.1	$^{2}H_2$-Cit	178.1→72.1	5	-13	-25	-28
Xle	132.1→86.1	$^{2}H_3$-Leu	135.1→89.1	5	-10	-12	-16
Lys	147.1→84.1	$^{13}C6$-$^{15}N_2$-Lys	155.1→90.1	10	-11	-18	-16
Met	150.2→61.2	$^{2}H_3$-Met	153.2→64.2	5	-11	-25	-26
Orn	133.2→70.2	$^{2}H_6$-Orn	139.2→76.2	10	-10	-23	-29

Table 2.2 (continued).

Phe	166.1→120.1	$^{13}C_6$-Phe	172.1→126.1	5	-12	-16	-12
Pro	116.2→70.2	$^{2}H_5$-Pro	121.2→74.2	5	-14	-18	-28
Ser	106.1→60.1	$^{13}C_3$-Ser	109.1→62.1	5	-12	-14	-24
Tyr	182.1→91.1	$^{13}C_6$-Tyr	188.1→142.1	5	-13	-15	-27
Val	118.1→72.2	$^{2}H_8$-Val	126.1→80.2	5	-14	-14	-14
C0	162.1→85	$^{2}H_9$-C0	171.1→85	5	-12	-23	-15
C2	204.1→85	$^{2}H_3$-C2	207.1→85	5	-10	-22	-14
C3	218.1→85	$^{2}H_3$-C3	221.1→85	5	-11	-23	-15
C4	232.2→85	$^{2}H_3$-C4	235.2→85	5	-12	-23	-15
C5	246.2→85	$^{2}H_9$-C5	255.2→85	5	-13	-23	-15
C5:1	244.2→85	$^{2}H_9$-C5	255.2→85	5	-13	-23	-15
C5DC	276.2→85	$^{2}H_3$-C5DC	279.2→85	10	-13	-25	-16
C5OH	262.2→85	$^{2}H_9$-C5	255.2→85	5	-13	-23	-15
C6	260.2→85	$^{2}H_3$-C6	263.2→85	5	-10	-24	-15
C8	288.2→85	$^{2}H_3$-C8	291.2→85	5	-11	-23	-15
C10	316.2→85	$^{2}H_3$-C10	319.2→85	5	-12	-25	-15
C10:1	314.2→85	$^{2}H_3$-C10	319.2→85	5	-12	-25	-15
C10:2	312.2→85	$^{2}H_3$-C10	319.2→85	5	-12	-25	-15
C12	344.2→85	$^{2}H_3$-C12	347.2→85	5	-13	-27	-14
C14	372.2→85	$^{2}H_9$-C14	381.2→85	5	-14	-28	-15
C14:1	370.2→85	$^{2}H_9$-C14	381.2→85	5	-14	-28	-15
C14:2	368.2→85	$^{2}H_9$-C14	381.2→85	5	-14	-28	-15
C16	400.3→85	$^{2}H_3$-C16	403.3→85	5	-15	-28	-15
C16OH	416.3→85	$^{2}H_3$-C16	403.3→85	5	-15	-28	-15

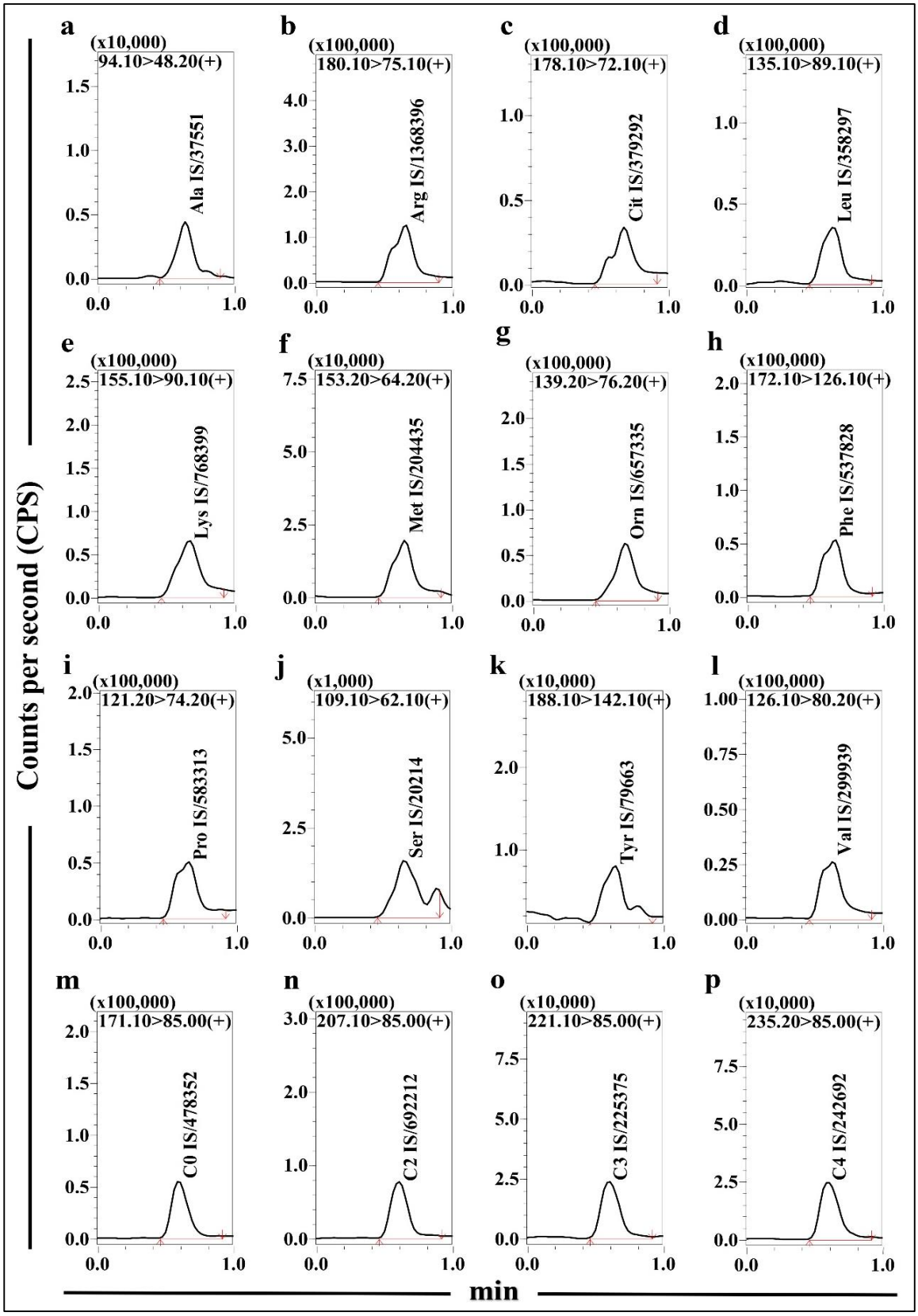
Table 2.2 (continued).

C16:1OH	414.3→85	$^{2}H_3$-C16	403.3→85	5	-15	-28	-15
C18	428.3→85	$^{2}H_3$-C18	431.3→85	5	-16	-30	-15
C18:1	426.3→85	$^{2}H_3$-C18	431.3→85	5	-16	-30	-15
C18OH	444.3→85	$^{2}H_3$-C18	431.3→85	5	-16	-30	-15
C18:1OH	442.3→85	$^{2}H_3$-C18	431.3→85	5	-16	-30	-15

Abbreviations: MRM, Multiple Reaction Monitoring (MRM) transitions: precursor ion → target ion; Ala, alanine; Arg, arginine; Cit, citrulline; Xle, (Leucine and isoleucine); Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Tyr, tyrosine; Val, valine; C0, free carnitine; C2, acetylcarnitine; C3, propionylcarnitine; C4 butyryl-/isobutyrylcarnitine; C5, isovaleryl-/2-methylbutyrylcarnitine, C5:1, tiglylcarnitine; C5DC, glutarylcarnitine; C5OH, hydroxy isovalerylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C10:1, decenoylcarnitine; C10:2, decadienoylcarnitine; C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C14:1, tetradecenoylcarnitine; C14:2, tetradecadienoylcarnitine; C16, palmitoylcarnitine; C16OH, hydroxy palmitoylcarnitine; C16:1OH, hydroxy hexadecenoylcarnitine; C18, stearylcarnitine; C18:1, oleylcarnitine; C18OH, hydroxy stearylcarnitine; C18:1OH, hydroxy oleylcarnitine.

2.4.5 Validation of the method

Validation of the method and performance of the Shimadzu LCMS 8050 (Shimadzu Corporation, Kyoto, Japan) was carried out using three levels of control specimens (Low, Medium and High) provided with the NeoMass AAAC kit (Labsystems Diagnostics Oy, Vantaa, Finland). The extractions of the analytes were done using previously described extraction method for the DBS cards and performance of the method was evaluated in terms of intra-assay and inter-assay precision, accuracy, linearity, limit of detection (LOD) or functional sensitivity, limit of quantitation (LOQ), and recovery. The method validation analysis was done for Ala, Arg, Cit, Leu, Lys, Met, Orn, Phe, Pro, Ser, Tyr, Val, C0, C2, C3, C4, C5, C6, C8, C10, C12, C14, C16, and C18. Fig 2.2 shows representative chromatograms generated from multiple reaction monitoring (MRM) of deuterated internal standards using LC-MS/MS. Representative chromatograms of amino acids and acylcarnitines measured from the low, medium, and high control DBS cards using LC-MS/MS has been shown in Fig 2.3, 2.4, and 2.5, respectively.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94.10>48.20(+)
Ala IS/37551
b
(x100,000)
180.10>75.10(+)
Arg IS/1368396
c
(x100,000)
178.10>72.10(+)
Cit IS/379292
d
(x100,000)
135.10>89.10(+)
Leu IS/358297
e
(x100,000)
155.10>90.10(+)
Lys IS/768399
f
(x10,000)
153.20>64.20(+)
Met IS/204435
g
(x100,000)
139.20>76.20(+)
Orn IS/657335
h
(x100,000)
172.10>126.10(+)
Phe IS/537828
i
(x100,000)
121.20>74.20(+)
Pro IS/583313
j
(x1,000)
109.10>62.10(+)
Ser IS/20214
k
(x10,000)
188.10>142.10(+)
Tyr IS/79663
l
(x100,000)
126.10>80.20(+)
Val IS/299939
m
(x100,000)
171.10>85.00(+)
C0 IS/478352
n
(x100,000)
207.10>85.00(+)
C2 IS/692212
o
(x10,000)
221.10>85.00(+)
C3 IS/225375
p
(x10,000)
235.20>85.00(+)
C4 IS/242692
Counts per second (CPS)
min

Fig 2.2 (continued).

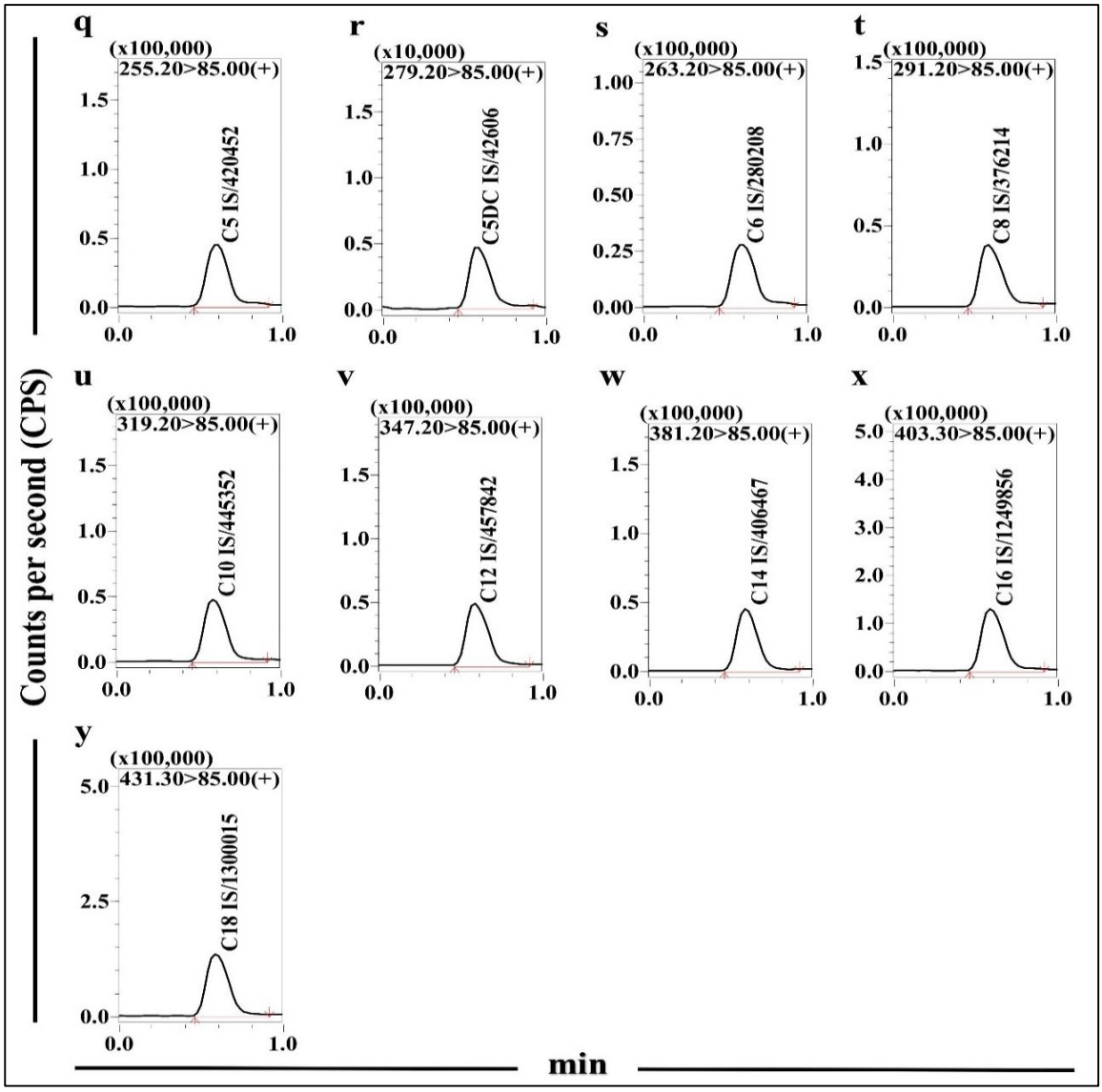
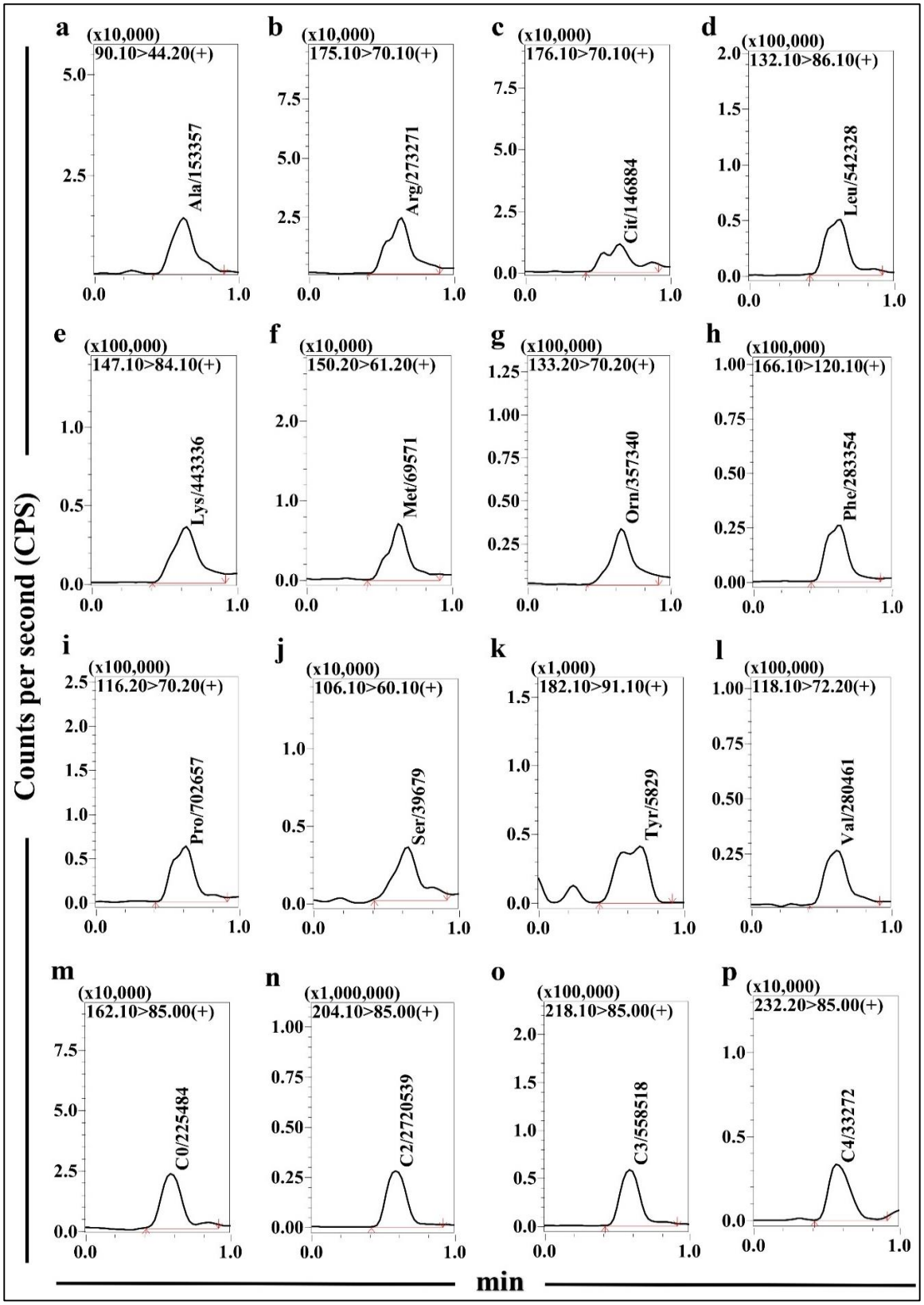


Fig 2.2: LC-MS/MS-generated representative multiple reaction monitoring (MRM) chromatograms for deuterated internal standards. The X-axis represents the time after a run in minute and Y-axis represents metabolites counted per second (CPS). The upper-left number inside each MRM chromatogram represents precursor ion followed by > sign and then the upper right number represents the target ion. Each metabolite detected has been shown vertically above the peak with a capital C followed by digit(s) and then IS for the internal standard. The concentration of each metabolite is proportional to the peak area. The internal standards are presented in the chromatogram as (a) $^{2}H_{4}$- Alanine (Ala IS), (b) $^{2}H_{4}$-^{13}C- Arginine (Arg IS), (c) $^{2}H_{2}$- Citrulline (Cit IS), (d) $^{2}H_{3}$- Leucine (Leu IS), (e) $^{13}C6$-$^{15}N_{2}$- Lysine (Lys IS), (f) $^{2}H_{3}$- Methionine (Met IS), (g) $^{2}H_{6}$- Ornithine (Orn IS), (h) $^{13}C_{6}$- Phenylalanine (Phe IS), (i) $^{2}H_{5}$- Proline (Pro IS), (j) $^{13}C_{3}$- Serine (Ser IS), (k) $^{13}C_{6}$- Tyrosine (Tyr IS), (l) $^{2}H_{8}$- Valine (Val IS), (m) $^{2}H_{9}$-free carnitine (C0 IS), (n) $^{2}H_{3}$-acetylcarnitine (C2 IS), (o) $^{2}H_{3}$-propionylcarnitine (C3 IS), (p) $^{2}H_{3}$-butyrylcarnitine (C4 IS), (q) $^{2}H_{9}$-Isovalerylcarnitine (C5 IS), (r) $^{2}H_{3}$-Glutarylcarnitine (C5DC IS), (s) $^{2}H_{3}$-Hexanoylcarnitine (C6 IS), (t) $^{2}H_{3}$-Octanoylcarnitine (C8 IS), (u) $^{2}H_{3}$-Decanoylcarnitine (C10 IS), (v) $^{2}H_{3}$-Lauroylcarnitine (C12 IS), (w) $^{2}H_{9}$-Myristoylcarnitine (C14 IS), (x) $^{2}H_{3}$-Palmitoylcarnitine (C16 IS), and (y) $^{2}H_{3}$-Stearoylcarnitin (C18 IS).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(x10,000)
90.10>44.20(+)
Ala/153357
b
(x10,000)
175.10>70.10(+)
Arg/273271
c
(x10,000)
176.10>70.10(+)
Cit/146884
d
(x100,000)
132.10>86.10(+)
Leu/542328
e
(x100,000)
147.10>84.10(+)
Lys/443336
f
(x10,000)
150.20>61.20(+)
Met/69571
g
(x100,000)
133.20>70.20(+)
Orn/357340
h
(x100,000)
166.10>120.10(+)
Phe/283354
i
(x100,000)
116.20>70.20(+)
Pro/702657
j
(x10,000)
106.10>60.10(+)
Ser/39679
k
(x1,000)
182.10>91.10(+)
Tyr/5829
l
(x100,000)
118.10>72.20(+)
Val/280461
m
(x10,000)
162.10>85.00(+)
C0/225484
n
(x1,000,000)
204.10>85.00(+)
C2/2720539
o
(x100,000)
218.10>85.00(+)
C3/558518
p
(x10,000)
232.20>85.00(+)
C4/33272
Counts per second (CPS)
min

Fig 2.3 (continued).

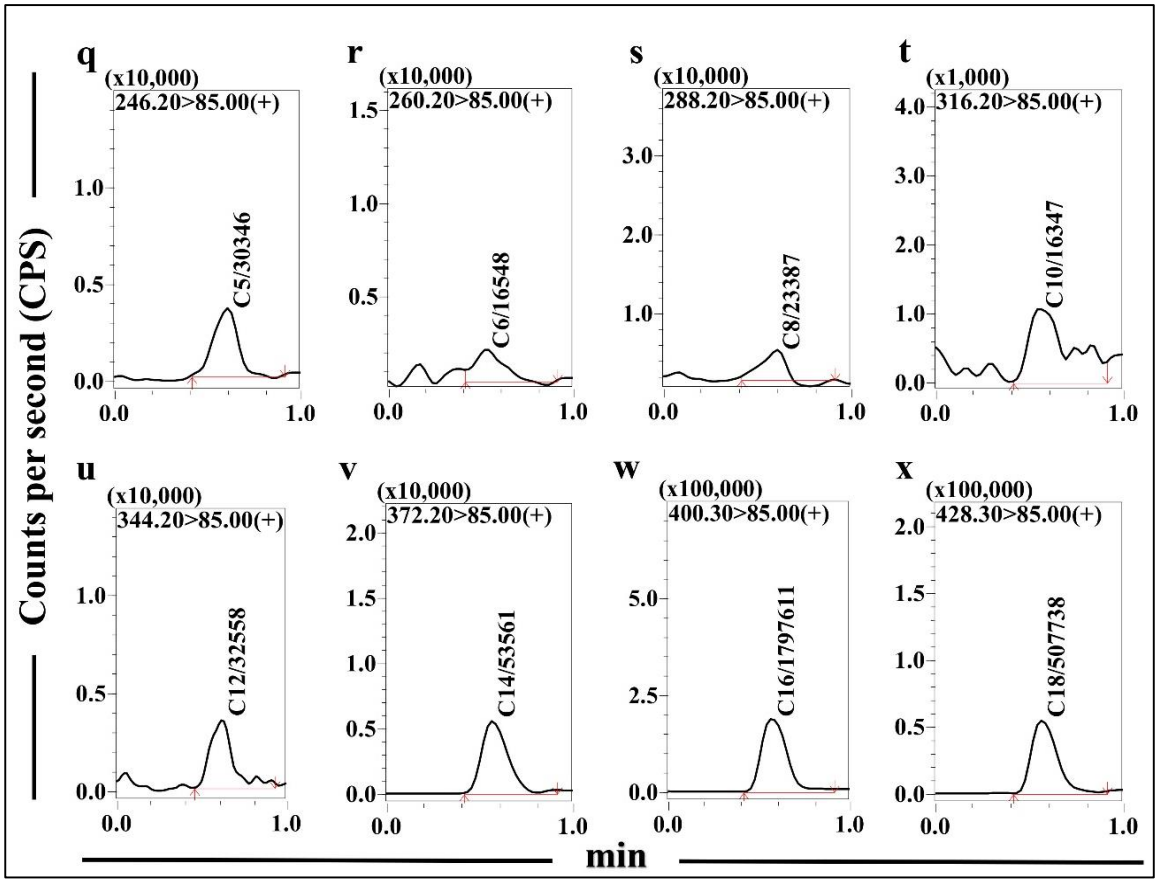


Fig 2.3: A representative chromatogram of amino acids and acylcarnitines measured from low control DBS cards using LC-MS/MS. The X-axis represents the time after run in minute and Y-axis represents metabolites counted per second (CPS). The upper-left number inside each MRM chromatogram represents a precursor ion followed by > sign and then the upper right number represents the target ion. Each metabolite detected has been shown vertically above the peak with the name of the specific analyte followed by a digit. The concentration of each metabolite is proportional to the peak area. The analytes are presented in the chromatogram as (a) Alanine (Ala), (b) Arginine (Arg), (c) Citrulline (Cit), (d) Leucine (Leu), (e) Lysine (Lys), (f) Methionine (Met), (g) Ornithine (Orn), (h) Phenylalanine (Phe), (i) Proline (Pro), (j) Serine (Ser), (k) Tyrosine (Tyr), (l) Valine (Val), (m) free carnitine (C0), (n) Acetylcarnitine (C2), (o) Propionylcarnitine (C3), (p) Butyrylcarnitine (C4), (q) Isovalerylcarnitine (C5), (r) Hexanoylcarnitine (C6), (s) Octanoylcarnitine (C8), (t) Decanoylcarnitine (C10), (u) Lauroylcarnitine (C12), (v) Myristoylcarnitine (C14), (w) Palmitoylcarnitine (C16), and (x) Stearoylcarnitin (C18).

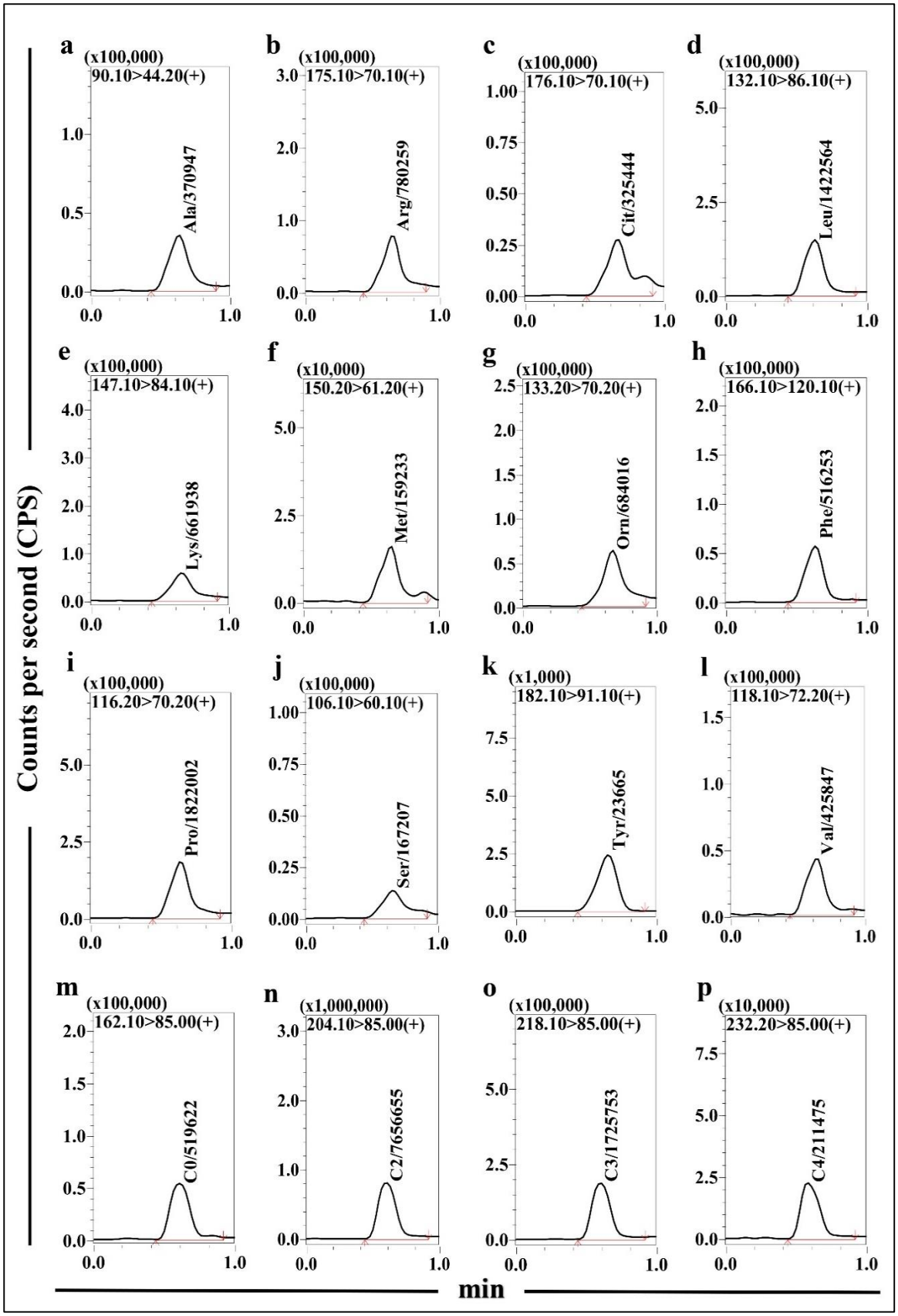

a
(x100,000)
90.10>44.20(+)
Ala/370947
b
(x100,000)
175.10>70.10(+)
Arg/780259
c
(x100,000)
176.10>70.10(+)
Cit/325444
d
(x100,000)
132.10>86.10(+)
Leu/1422564
e
(x100,000)
147.10>84.10(+)
Lys/661938
f
(x10,000)
150.20>61.20(+)
Met/159233
g
(x100,000)
133.20>70.20(+)
Orn/684016
h
(x100,000)
166.10>120.10(+)
Phe/516253
i
(x100,000)
116.20>70.20(+)
Pro/1822002
j
(x100,000)
106.10>60.10(+)
Ser/167207
k
(x1,000)
182.10>91.10(+)
Tyr/23665
l
(x100,000)
118.10>72.20(+)
Val/425847
m
(x100,000)
162.10>85.00(+)
C0/519622
n
(x1,000,000)
204.10>85.00(+)
C2/7656655
o
(x100,000)
218.10>85.00(+)
C3/1725753
p
(x10,000)
232.20>85.00(+)
C4/211475
Counts per second (CPS)
min

Fig 2.4 (continued).

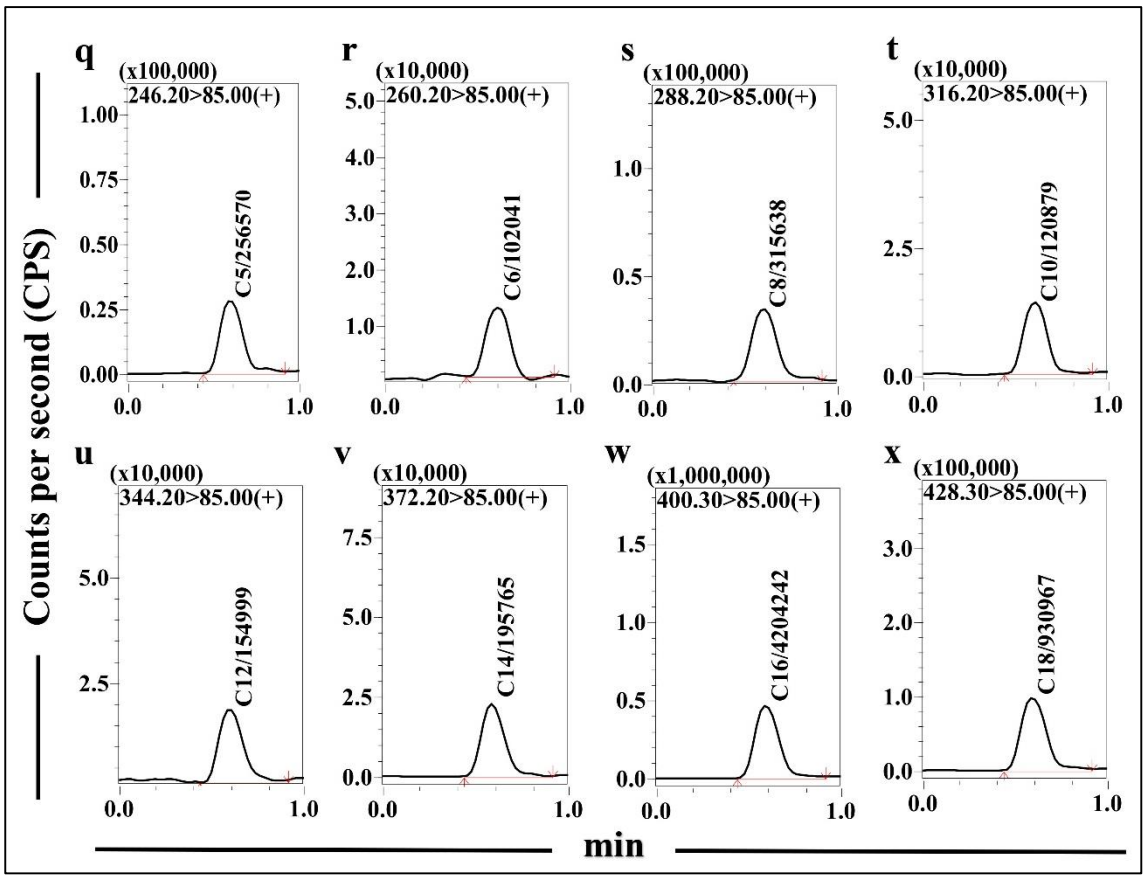
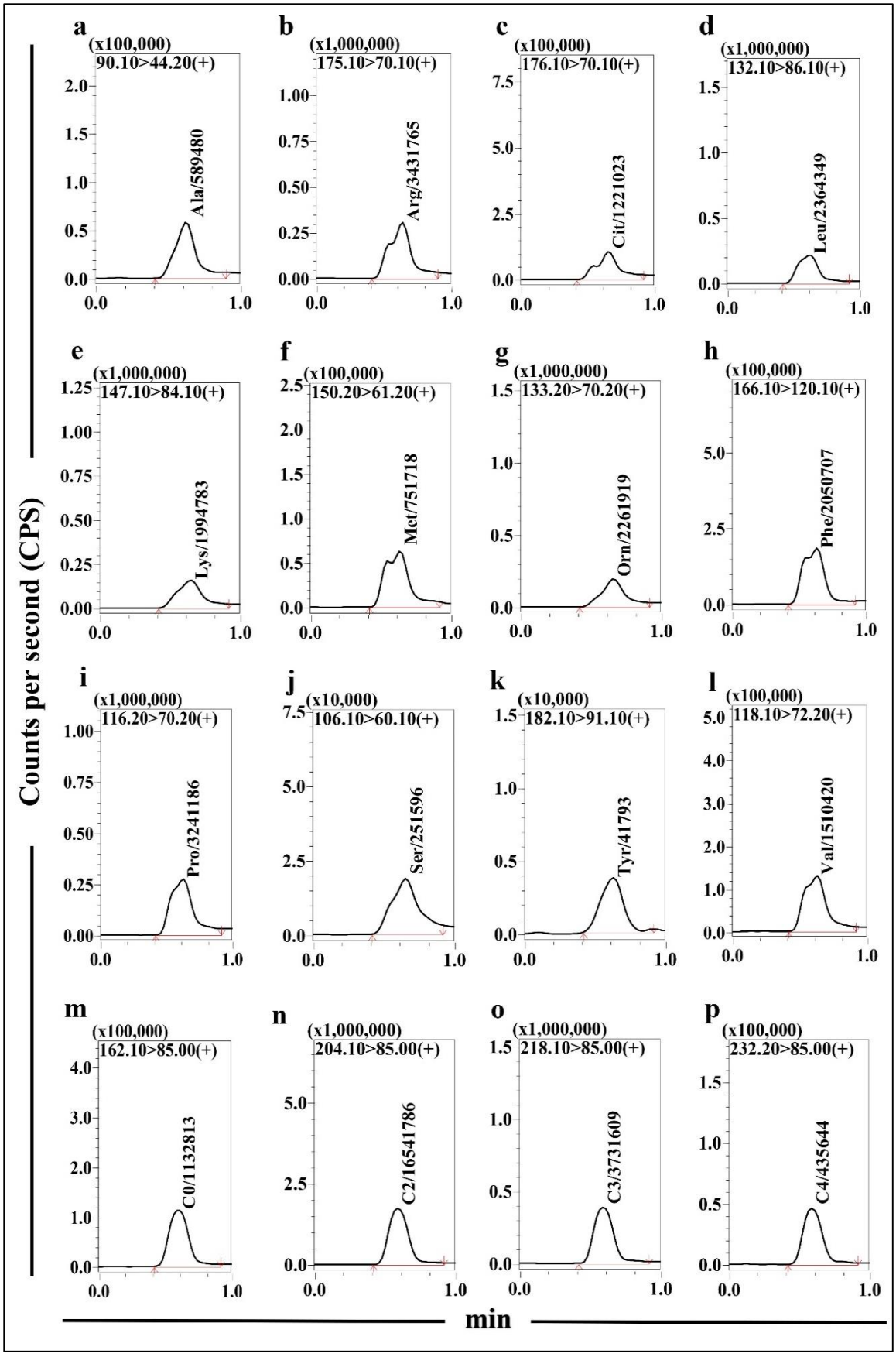


Fig 2.4: A representative chromatogram of amino acids and acylcarnitines measured from medium control DBS card using LC-MS.MS. The X-axis represents time in minute and Y-axis represents the intensity of analytes in counted per second (CPS). The upper-left number inside the MRM chromatogram represents precursor ion followed by > sign and then the upper right number represents the target ion. Each analyte detected has been shown vertically above the peak with the name of the analyte followed by area of peak. The concentration of each metabolite is proportional to the peak area. Abbreviations are listed in the legend of Fig 2.3.


a
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Ala/589480
b
(x1,000,000)
175.10>70.10(+)
Arg/3431765
c
(x100,000)
176.10>70.10(+)
Cit/1221023
d
(x1,000,000)
132.10>86.10(+)
Leu/2364349
e
(x1,000,000)
147.10>84.10(+)
Lys/1994783
f
(x100,000)
150.20>61.20(+)
Met/751718
g
(x1,000,000)
133.20>70.20(+)
Orn/2261919
h
(x100,000)
166.10>120.10(+)
Phe/2050707
i
(x1,000,000)
116.20>70.20(+)
Pro/3241186
j
(x10,000)
106.10>60.10(+)
Ser/251596
k
(x10,000)
182.10>91.10(+)
Tyr/41793
l
(x100,000)
118.10>72.20(+)
Val/1510420
m
(x100,000)
162.10>85.00(+)
C0/1132813
n
(x1,000,000)
204.10>85.00(+)
C2/16541786
o
(x1,000,000)
218.10>85.00(+)
C3/3731609
p
(x100,000)
232.20>85.00(+)
C4/435644
Counts per second (CPS)
min

Fig 2.5 (continued).

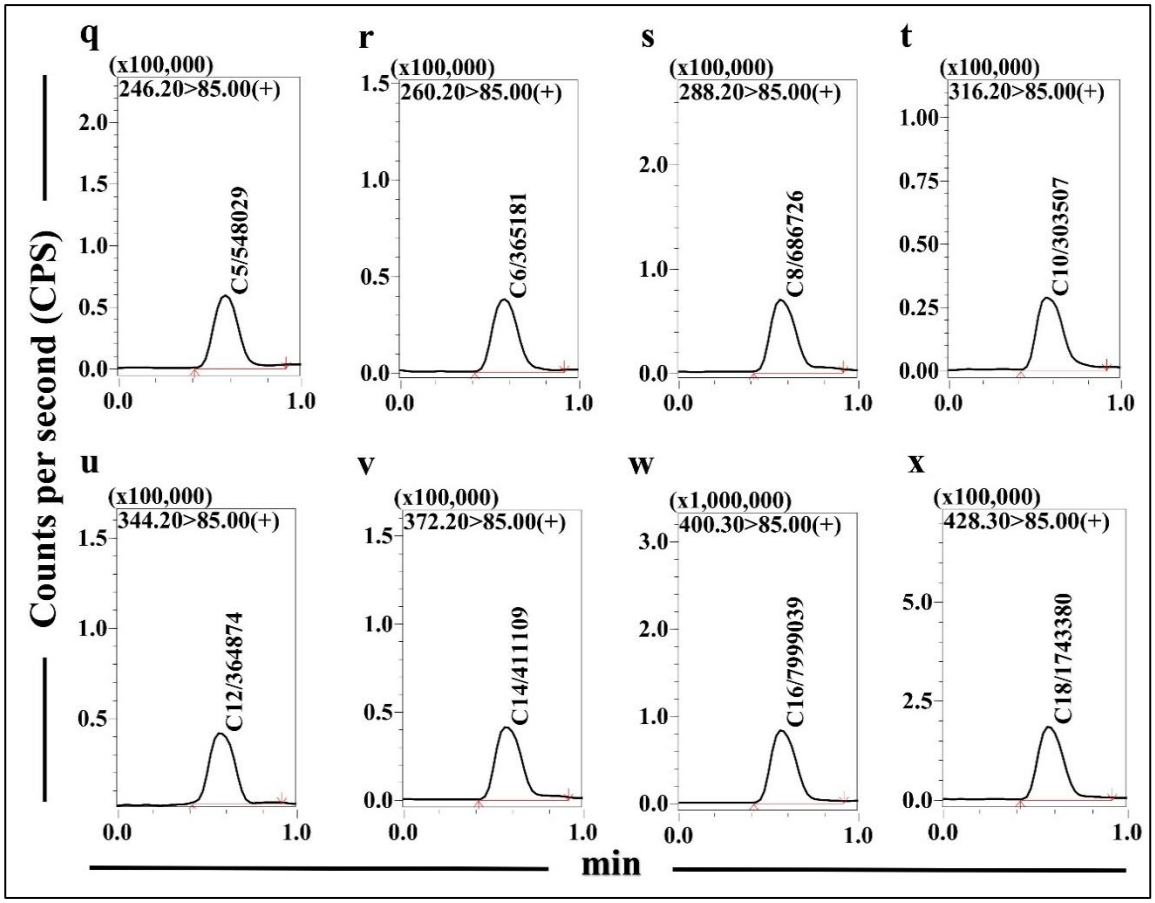

Fig 2.5: A representative chromatogram of amino acids and acylcarnitines measured from high control DBS cards using LC-MS.MS. The X-axis represents time in minute and Y-axis represents the intensity of analytes in counted per second (CPS). The upper-left number inside each MRM chromatogram represents precursor ion followed by > sign and then the upper right number represents the target ion. Each analyte detected has been shown vertically above the peak with name of analytes followed by the area of peak. The concentration of each metabolite is proportional to the peak area. Abbreviations are listed in the legend of Fig 2.3.

2.5. GC-MS analysis for urinary metabolic profiling

GC-MS (second-tier test) was used for the urinary metabolic screening test. Urine specimens of the patients who were detected as positive by LC-MS/MS analysis were analyzed at NeoCare Diagnostics Pvt. Ltd., Mumbai, India.

2.6. Qualitative screening of G6PD deficiency using fluorescent spot test (FST)

G6PD qualitative screening was performed using a modified fluorescent spot test (FST) developed by Butler and Mitchell where the $NADP^+$ was reduced by the G6PD enzyme to NADPH, which gives fluorescence at 340 nm. The enzyme activity was determined by measuring the rate of fluorescence due to the reduction of $NADP^+$ [75].

$$\text{Glucose-6-phosphate} + NADP^{+} \xrightarrow{\text{G6PD}} \text{Gluconate-6-phosphate} + NADPH$$

2.6.1 Procedures of FST test

Whole blood (10.0 µL) was mixed with 100.0 µL of screening solution (0.001 M glucose-6-phosphate, 0.00075 M NADP+, 0.2% saponin, 0.225 M Tris-HCl buffer pH 7.4, 0.0008 M GSSG) and made blood spots on a filter paper (Double Ring 102, Hangzhou Xinhua Paper Industry Co., Ltd, Zhejiang, China) at intervals of 0, 5, 10, 15 minutes, followed by air-drying at room temperature for 2 hours. After drying, the fluorescence intensity of each spot was read using Gel Doc™ XR^{+} (BioRad, CA, USA) gel documentation system. Each test was paired with a participant's specimen and a control specimen from a known G6PD-deficient participant. The participant's specimens were considered as normal if there was any presence of fluorescence spot within 5 minutes after initiation of the test. Spots without any fluorescence were considered fully deficient. Although the diagnosis of G6PD deficiency by FST is most commonly done by reading fluorescence after 10 minutes, the incubation time was increased up to 15 minutes to distinguish between moderate or intermediate deficiency.

2.7. Quantitative G6PD activity assay

Randox G6PD assay kit (Randox Laboratories Ltd., Crumlin, UK) was used to measure G6PD enzyme activity and the manufacturer's instruction was followed. G6PD enzyme activity was measured quantitatively by measuring an increase in absorbance of NADPH at 340 nm produced in the reaction catalyzed by the enzyme [76].

2.7.1. Procedures of G6PD enzyme activity assay

The hematocrit value of each specimen was measured by a capillary Centurion Scientific C2 series centrifuge machine (Centurion Scientific, Chichester, UK). Blood hemoglobin (Hb) level was measured empirically from the hematocrit value and the method of Lee *et al.* was followed [77]. G6PD enzyme activity at ambient temperature ~25˚C was measured in U/g Hb using the formula provided in the Randox G6PD assay kit, (33650 x ΔA 340 nm/min x 100) / Hb (g/dL). To obtain the enzyme activity at 37˚C, the calculated enzyme activity at 25˚C was multiplied by the temperature correction factor 2.076.

The G6PD enzyme was assayed spectrophotometrically and each sample was measured in duplicate. The experiment was repeated provided that there was any discrepancy between

the two readings of each sample. Only the adjusted male median value of G6PD enzyme activity was used to define the G6PD deficiency in both males and females. The female participants were excluded to calculate the adjusted median value to minimize the impact of heterozygosity on the definition of G6PD activity, according to the method described by Domingo *et al.* [78]. The participants who had enzyme activity level <60% of the adjusted male median were as taken as deficient for both male and female participants.

2.8 Reticulocyte counts

The light microscope was used to count the reticulocytes after supravital staining by new methylene blue (St. Louis, USA). A total of 1000 erythrocytes including reticulocytes were counted to calculate the percentages of reticulocytes in the total RBCs.

2.9 DNA Extraction

Genomic DNA (gDNA) from whole blood was extracted according to guidelines of the QIAGEN flexigene® DNA kit (QIAGEN, Hilden, Germany) manual.

2.9.1 Procedures of DNA isolation

For DNA isolation, FG2/QIAGEN Protease mixture (Denaturation buffer) was prepared by adding QIAGEN Protease with FG2 buffer in 1:100 ratio. This mixture was used within 1 hour of preparation. FG1 buffer (500 µL) was taken in a 1.5 mL microcentrifuge tube and 200 µL whole blood was mixed with FG1 buffer by inverting the tube 20 times. The tube was centrifuged for 5 minutes at 10,000 x g using Heraeus™ Fresco™ 21 Microcentrifuge (Thermo Scientific™, USA). The supernatant was discarded and the tubes were was kept inverted on a clean piece of tissue paper for 2 minutes, taking care that the pellet would remain in the tube. FG2/QIAGEN Protease buffer (100 µl) was added and vortexed immediately to homogenize the pellet completely. The tube was then centrifuged for 3–5 seconds. After centrifugation, the tube was placed in a water bath at 65ºC for 5 minutes. After incubation, 100 µL isopropanol (100%) was added and mixed thoroughly by inversion (at least 20 times) until DNA becomes visible as threads or a clump. Then the tube was centrifuged for 3 minutes at 10,000 x g. The supernatant was discarded and the tube was left inverted on a clean tissue paper for 5 minutes, making sure that the pellet was in the tube. The mixture was vortexed for 5 seconds after the addition of 75% Ethanol (100 µL). The tube was then centrifuged for 3 minutes at 10,000 x g. The supernatant was discarded and the tube was inverted on a clean piece of tissue paper for at least 5 minutes,

taking care that the pellet remained in the tube. The pellet was air-dried until all the liquid had evaporated. Nuclease-free water (50 µL) was added to dissolve the DNA pellet.

2.10. RNA isolation

RNA extraction was carried out using Trizol® LS (Life Technologies, CA, USA) from whole blood according to the manufacturer's instructions and described briefly below.

2.10.1. Procedures of RNA extraction

For RNA extraction from whole blood, 750 µL Trizol® LS reagent was mixed with 125 µL of whole blood specimen and 125 µL nuclease-free water into a micro-centrifuge tube. The tube was inverted several times to homogenize the specimen and incubated at room temperature for 5 minutes. The tube was centrifuged at 12,000 × g at 4°C for 5 minutes. The clear supernatant was transferred into a new 1.5 mL microcentrifuge tube. Chloroform (200 µL) was added and the tube was incubated for 2–3 minutes at room temperature. The tube was then centrifuged for 15 minutes at 12,000 × g at 4°C and the colorless upper aqueous phase containing RNA was transferred into a new 1.5 mL microcentrifuge tube. Isopropanol (500 µL) was added to the aqueous phase and incubated for 10 minutes at room temperature. After centrifugation at 12,000 × g at 4°C, the supernatant was discarded and the RNA pellet was washed with 70% ethanol. The supernatant was discarded after centrifugation at 7500 × g at 4°C for 5 minutes. Nuclease-free water was added to the dried RNA pellet and stored at -70°C.

2.11. cDNA preparation

Extracted total RNA was subjected to reverse transcription using SuperScript® III First-Strand Synthesis System (Invitrogen, CA, USA) according to the manufacturer's protocol. The synthesis of cDNA from mRNA was carried out using reverse transcriptase enzyme and random hexamer primer.

2.11.1. Procedures of cDNA preparation

RNA (500 ng) was incubated at 65°C for 5 minutes in a final volume of 10 µL containing 1µL of dNTPs (10mM), 1 µL of hexamer primer (50 ng/µL), and nuclease-free water (up to 10µL). A second mixture called cDNA synthesis mixture (10 µL) containing 2 µL of 10X RT Buffer, 4 µL of $MgCl_2$ (25 mM), 2 µL of DTT (0.1 mM), 1 µL of RNaseOut, and 1 µL of SuperScript III RT enzyme was prepared. cDNA synthesis mixture (10 µL) was added into the first mixture and incubated using a thermal cycler (BioRad, USA). The

thermal cycler was programmed to maintain 25˚C for 10 minutes, 50˚C for 50 minutes, and 85˚C for 5 minutes for inactivation of reverse transcriptase enzyme. After incubation, 1 µL of RNase H was added to the reaction mixture and the tube was incubated again for 20 minutes at 37°C. The cDNA was stored at -20˚C freezer for the polymerase chain reaction.

2.12. Polymerase chain reaction (PCR)

The extracted gDNA and cDNA were used as templates for polymerase chain reaction (PCR) to generate millions of copies of a particular gene for Sanger's DNA sequencing.

2.12.1 Procedures of PCR

2.11.1.1 Primer designing

Six pairs of primer which together cover all 13 exons, some of the flanking introns, and also 5´ and 3´ untranslated regions (UTRs) of the G6PD gene, were designed for mutation analysis. The primer sequences provided in Table 2.3 were used in the study.

Table 2.3: Primer sequences for polymerase chain reactions.

Sl. No.	Primer	Nucleotide sequence (From 5´ to 3´)	Primer size (nucleotides)
1	**Ex1F**	AAGCCGGCGAGAAGTGTGAGG	21
2	**Ex6R**	GCACCATGAGGTTCTGCACCAT	22
3	**Ex5F**	CTACGAGGCCGTCACCAAGAAC	22
4	**Ex10R**	GATCACCAGCTCGTTGCGCTTG	22
5	**Ex9F**	CACTTTTGCAGCCGTCGTCCTC	22
6	**Ex13R$_1$**	GTGCAGCTGAGGTCAATGGTCC	22
7	**Ex13F**	GGGTTTCCAGTATGAGGGCACC	22
8	**Ex13R$_2$**	GGGCTGTTTGCGGATTTAATGG	22
9	**UTR5´ TV2F**	GCTCCGAGAAAGTCCCAGTTTC	22
10	**UTR5´ TV2R**	GCCCCTACTGTCCGGTTTCC	20
11	**UTR5´ TV1F**	TGGGGATGCGGGAGCACTAC	20
12	**UTR5´ TV1F**	CAAGAGAGGAGGTGCGGGGTAT	22

2.12.1.2 PCR reaction

PCR was performed in a final reaction volume of 20.0 µL containing 2.0 µL of 10X PCR buffer (with 15.0 mM $MgCl_2$), 0.5 µL $MgCl_2$ (25 mM), 4.0 µL Q-solution (Qiagen), 3.2 µL dNTPs mixture (2.5 mM), 0.4 µL forward (10 mM) and 0.4 µL reverse primers (10 mM), 0.2 µL of HotStarTaq DNA polymerase (Qiagen) and 200.0 ng of genomic DNA or 2.0 µL of cDNA preparation and finally total volume was made to 20.0 µL with nuclease-free water. Thermal cycling profile for forward primer Ex1F and reverse prime Ex6R was

as follows: pre-denaturation at 95˚C for 15 minutes; 35 cycles of denaturation at 94˚C for 35 seconds, annealing at 63˚C for 40 seconds and extension at 72˚C for 1 minute 10 seconds; and a final extension at 72˚C for 10 minutes. The same thermal cycling profile was followed for forward primer Ex5F and reverse primer Ex10R. PCR was done using cDNA as a template for these primer sets.

For forward primer Ex9F and reverse primer Ex13R1 as well as for forward primer Ex13F and reverse primer Ex13R2, the following cycling profile was used: pre-denaturation at 95˚C for 15 minutes; 35 cycles of denaturation at 94˚C for 45 seconds, annealing at 59˚C for 30 seconds and extension at 72˚C for 1 minute 20 seconds; and a final extension at 72˚C for 10 minutes. Genomic DNA was added as the template for this PCR amplification.

To amplify the 5´ untranslated region (5´UTR) from transcript variant-2, forward primer TV2F and reverse primer TV2R were used. On the other hand, forward TV1F and reverse TV1R primers were used to amplify 5´UTR from transcript variant-1. Both primer sets had the same thermal cycling profile as follows: pre-denaturation at 95˚C for 15 minutes; 35 cycles of denaturation at 94˚C for 45 seconds, annealing at 59˚C for 35 seconds and extension at 72˚C for 40 seconds; and a final extension at 72˚C for 10 minutes. Genomic DNA was amplified as a template for this PCR reaction.

2.13. PCR product purification

PCR amplified products were purified for Sanger's sequencing using a MiniElute PCR product purification system (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.13.1 Procedures of PCR product purification

PCR product was transferred into a 1.5 mL microcentrifuge tube and PB buffer (5 volume of PCR product) was added followed by mixing. After the addition of 3M sodium acetate (10 µL, pH 5.0) the content was transferred to the MiniElute column along with a 2 mL collection tube. The tube was centrifuged at 17900 x g for 1 minute and the flow-through was discarded. The column was placed back into the collection tube and 750 µL Buffer PE was added into the MinElute Column. After centrifugation at 17900 x g for 1 minute, the flow-through was discarded. The column was again placed in a new collection tube and centrifuged at 17900 x g for 1 minute to remove the residual ethanol. After centrifugation, the column was placed into a 1.5 mL microcentrifuge tube and 15-20 µL nuclease-free water was added. The column along with the tube was centrifuged at maximum speed for

1 minute to elute the DNA. This DNA containing solution was checked by agarose gel electrophoresis before sequencing.

2.14. Agarose gel electrophoresis

Purified PCR products were checked by running the samples on a 1% agarose gel.

2.14.1 Procedures of agarose gel electrophoresis

To check the quality of the purified products using agarose gel electrophoresis, purified PCR products (5 µL) were mixed with 6X Loading Dye (1 µL) and loaded into the well of agarose gel using a micropipette. To estimate the size of the PCR products, 1 µL of 1 kb Plus DNA ladder (Invitrogen™, USA) was also loaded with the purified PCR products. Electrophoresis was done using 1X TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH: 8.0) at 100 V for 1 hour. The bands of the PCR products were subsequently visualized using the Gel Doc™ XR+ (BioRad, USA) gel documentation system.

2.15. Sequencing of purified PCR product

Purified PCR products were further subjected to cycle sequencing using BigDye Version 3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. After cycle sequencing, the products were purified using BigDye XTerminator™ purification kit (Applied Biosystems) according to the manufacturer's protocol. The purified cycle sequencing products were then subjected to capillary electrophoresis using POP-6 (Applied Biosystems, USA) on an ABI PRISM 310 Automated Sequencer (Applied Biosystems, USA).

2.15.1 Procedures of Sanger's DNA sequencing

2.15.1.1 Cycle sequencing

The cycle sequencing reaction was performed in a final reaction volume of 10.0 µL containing 4.0 µL of BigDye™ Terminator 3.1 Ready Reaction mix, 1 µL of forward (3.2 µM) or reverse primer (3.2 µM), template DNA (10-50 ng), and nuclease-free water (up to 10 µL). Cycle sequencing was done using a thermal cycler (BioRad, USA) and the thermal cycling profile as follows: initial incubation at 96˚C for 1 minute; 35 cycles of denaturation at 96˚C for 10 seconds, annealing at 58˚C for 5 seconds and extension at 60˚C for 4 minutes; and finally hold at 4˚C until purification.

2.15.1.2. Purification of cycle sequencing products

To purify the products after cycle sequencing, 45 µl SAM solution was added into each well for 10 µl cycle sequencing PCR product. Xterminator solution (10 µL) was added in each well and the PCR tube was vortexed at 2500 rpm for 30 minutes. The tube was centrifuged at 1000 x g for 2 minutes and the supernatant was kept in an 8 well strip which was then placed on the ABI Prism 310 capillary (Applied BioSystems, USA) automated sequencer for DNA sequencing.

2.15.1.3. Sequencing data collection and mutation identification

Sequencing data was collected using an ABI PRISM 310 data collection software version 3.1.0 (Applied Biosystems). The collected FASTA format of the sequencing data was used to identify substituted base(s) by aligning query sequence with wild type sequence on the NCBI database by using the Basic Local Alignment Search Tool (BLAST). The ExPASy translate tool was used to convert the nucleotides sequence into corresponding amino acids. Amino acid sequences from deficient participants were aligned in ClustalW tool with the reference sequence (NM_001042351.2) to find the substituted amino acid.

2.16. Real-time PCR-high resolution melting (HRM) curve analysis

The real-time PCR followed by HRM analysis was performed on a CFX96 Touch™ Real-Time PCR machine (BioRad, USA). A technique called high-resolution melt (HRM) curve analysis was used to detect the change of the sequence of amplified PCR products. This real-time PCR system can be used for the detection of mutations, polymorphisms, and epigenetic differences in double-stranded DNA (dsDNA) samples. For HRM analysis, PCR amplification of the region of interest was done using a specific set of primers in the presence of a dsDNA binding dye such as SYBR green. After PCR amplification, the PCR product was heated from around 60°C to around 95°C. At the melting point of the amplicon, the dsDNA separates and changes of the fluorescence level are monitored in real-time. The real-time PCR instrument generates a graph by plotting the intensity of fluorescence against temperature. As the melting point of the specific PCR product depends on the DNA sequence, the curve generated for the wild type DNA can be differentiated from the mutated DNA from the appearances of the two melt curves. In case of the heterozygous condition, the melt curve was showing different shape from homozygote melt curves due to the presence of heteroduplexes (base pair mismatches) in the PCR reaction. Fig 2.6 shows that

the melt curves of two different amplicons (wild type and mutated) can be used to identify the mutation.

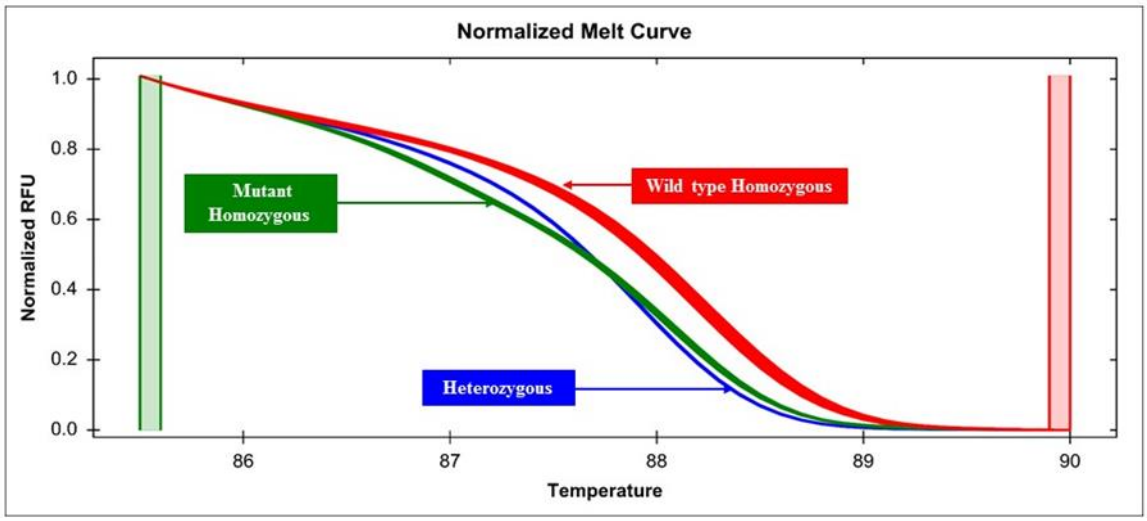

Fig 2.6: Level of fluorescence (Normalized RFU) versus temperature plot generated from the high-resolution melt (HRM) analysis. The Red-colored curves are generated from the samples with wild type DNA (homozygous), the green curves are generated from the samples with mutant DNA (homozygous), and the blue-colored curves are generated from samples with heterozygous DNA.

2.16.1 Procedures of HRM analysis

2.16.1.1 Primer design

For screening of G6PD Orissa (c.C131G) variant, PCR amplification was done using forward primer 5′-CACCTGTTCCCTCTGCCAC-3′and reverse primer 5′-TACCAGAT GGTGGGGTAGATC-3′ that spans a 62 bp amplicon of G6PD gene. On the other hand, G6PD Kalyan-Kerala variant was screened by targeting a 226 bp sequence of the G6PD gene using the forward primer 5′-CCCAACTCAACACCCAAGGA-3′ and the reverse primer 5′-CTCATTCTCCACATAGAGGACGAC-3′ [74]. For screening of other G6PD variants including c.A95G, c.C274T, c.G392 T, C406T, c.G487A, c.A493G, c.T517C, c. C519G, c.C563T, c.C592T, c.A835G, c.G871A, c.C1024T, c.C100 4T, c.G1340 T, c.C1360T, c.G1376 T, c.G1381A and c.G1388A, PCR amplification was performed using previously published primers [74]. The lengths of the amplified product ranged from 62 bp to 226 bp, which were within the limit of standard amplicon size for HRM analysis [79, 80]. Table 2.4 shows the primer sequences used for PCR amplification to detect the mutations using HRM-based approach.

Table 2.4: Primer sequences for high-resolution melt (HRM) analysis of G6PD gene mutation.

Sl. No.	Mutation detection by HRM	Primer	Nucleotide sequence (From 5´ to 3´)	Product size (bp)
1	G6PD Orissa	Forward	CACCTGTTCCCTCTGCCAC	62
		Reverse	TACCAGATGGTGGGGTAGATC	
2	G6PD Kalyan-Kerala	Forward	CCCAACTCAACACCCAAGGA	226
3		Reverse	CTCATTCTCCACATAGAGGACGAC	
4	c.A95G[a]	Forward	GGCGATGCCTTCCATCAGTC	109
		Reverse	AGGCATGGAGCAGGCACTTC	
5	c.C274T[a]	Forward	TGTGTCTGTCTGTCCGTGTCTC	108
		Reverse	AGGCTGCATCATCGTACTGG	
6	c.G392 T[a] C406T[a]	Forward	TACCAGCGCCTCAACAGC	77
		Reverse	GGCAAGGCCAGGTAGAAGA	
7	c.A493G[a] c.T517C[a] c. C519G[a]	Forward	GCAGCTCTGATCCTCACTCC	137
		Reverse	GGTTGGACAGCCGGTCA	
9	c.C592T[a]	Forward	TGTTCCGTGAGGACCAGATCTA	64
		Reverse	AGGTTCTGCACCATCTCCTTG	
10	c.A835G[a]	Forward	CGTGATGCAGAACCACCTACT	92
		Reverse	CCTTCTCATCACGGACGTCA	
11	c.G871A[a]	Forward	CCCAACTCAACACCCAAGGA	86
		Reverse	TGGCCTGCACCTCTGAGAT	
12	c.C1024T[a]	Forward	CACTTTTGCAGCCGTCGT	65
		Reverse	CTCGAAGGCATCACCTACCA	
13	c.C1004T[a]	Forward	CCAAAGGGTACCTGGACGAC	86
		Reverse	CTCATTCTCCACATAGAGGACGAC	
14	c.C1360T[a]	Forward	GCCTCATCCTGGACGTCTTC	92
		Reverse	CCCATAGCCCACAGGTATGC	
15	c.G1376 T[a] c.G1381A[a] c.G1388A[a]	Forward	GCCCTCCCTCCCTGTGTG	111
		Reverse	CAGCTCAATCTGGTGCAGCAGT	

[a]Primer sequence for PCR amplification was performed using previously published primers [74].

2.16.1.2 Real-time PCR amplification

A reaction volume of 10 µL was used for real-time PCR. The composition of PCR mixture was as follows: 5 µL of 2X precision melt supermix (BioRad), 0.2 µL of forward primers (10 µM), 0.2 µL of reverse primers (10 µM), 50 ng of genomic DNA in a total of 10 µL reaction volume. The thermal cycling profile for the real-time PCR was as follows: initial denaturation at 95°C for 3 minutes; 40 cycles of PCR amplification with denaturation at 94°C for 10 seconds followed by primer annealing at 58°C for 30 seconds and then

extension at 72°C for 30 seconds. The final extension of PCR product was performed at 72°C for 5 minutes. After completion of PCR, the subsequent melt curve program had the following steps: denaturation at 95°C for 30 seconds, renaturation at 60°C for 30 seconds, and then melting at 60°C to 95°C with an increment of 0.1°C per 5 seconds. Real-time HRM analysis was performed by parallel testing of (a) specimens of the present study, (b) sequenced wild type reference samples, and (c) available homozygous/hemizygous samples for G6PD Orissa (c.C131G), G6PD Mahidol (c.G487A) and G6PD Kalyan-Kerala (c.G949A), which were previously detected in this study [81].

2.17. Statistical analysis

GraphPad Prism 7 software (GraphPad Software, Inc., USA) was used for statistical analysis. Unpaired two-tailed t-test with Welch's correction was performed for comparisons of Hb concentrations and reticulocyte counts between deficient and non-deficient participants. Percentiles, mean, standard deviations (SD), coefficient of variance (%CV) and relative error (RE %) were calculated using standard statistical formula.

RESULTS

CHAPTER 3

3. Results

3.1 Establishment of cut-off values of amino acids and acylcarnitines and screening of suspected patients with IEM

3.1.1 Validation of LC-MS/MS method for the quantitation of amino acids and acylcarnitines

To check intra-assay and inter-assay variability of the method, three levels of control specimens, namely low, medium and high were run in 15 different replicates over a period of 5 days for each analyte and the analysis was done according to the CLSI EP5-A2 guideline [82]. Table A1 (Appendix I) shows that the average range of intra-assay percentage of coefficient of variation (%CV) of 11 amino acids (Alanine, Arginine, Citrulline, Leucine, Lysine, Methionine, Ornithine, Phenylalanine, Proline, Serine, Tyrosine, Valine) was within 20% of the target value which was within the acceptable limit [83, 84]. However, tyrosine showed 23.15% intra-assay %CV. The %CV of free carnitine (C0) and the acylcarnitines including C2, C3, C4, C5, C8, C10, C12, C14, C16, and C18 was also within the acceptable limit (20%), except for C6 (36.47%) which might be due to very low concentrations of the analyte present in the low level controls which were provided with the kit (0.13 µmol/L). The accuracy of the method was determined as the percentage of relative error (RE%). In the case of intra-assay accuracy, the average ranges of RE% of amino acids and acylcarnitines were -19.85 to +9.33% and -6.46 to +6.93%, respectively, which were within the acceptable ranges (Appendix I: Table A1) [84].

The average ranges of inter-assay %CV for amino acids and acylcarnitines were 1.32%–11.60% and 1.16–14.14%, respectively. The average ranges of inter-assay RE% for amino acids and carnitine-acylcarnitines were -19.31 to +3.55 % and -6.61 to +8.36%, respectively. These data demonstrated that the accuracy and precision of inter-assay were within acceptable limits (Appendix I: Table A2) [83, 84]. The ranges of average recovery rates for amino acids and carnitine-acylcarnitines were 80.68–103.54% and 93.37–108.35%, respectively (Appendix 1: Table A3) and they were within acceptable limits [84]. The linearity of the method for the quantitation of each analyte was evaluated following CLSI-EP06 guideline [85]. As shown in Table A3 (Appendix I), the coefficient of determination ($R^2 > 0.99$) along with slope and y-intersect indicated that the method was linear for all concentration levels (low, mid, and high) of each analyte. LOD and LOQ for all analytes were also calculated following CLSI-EP17 guideline, and the results have been demonstrated in Appendix I (Table A3) [86]. Together, the data generated for the method

validation study indicated that this method was suitable for the quantitation of amino acids and acylcarnitines from the DBS specimens [83, 84].

3.1.2. Determination of cut-off values of amino acids, acylcarnitines, and marker ratios

In this study, the cut-off values were calculated using the percentile distribution of metabolites in healthy subjects according to CLSI C28-A2 guideline [87]. LC-MS/MS-based determination of blood concentrations of 12 amino acids, free carnitine, and 23 acylcarnitine species were determined and the ratios of metabolites (12 amino acid ratios and 18 ratios for acylcarnitines) were calculated. The 2.5^{th}, 50^{th} (median), and 97.5^{th} percentiles were calculated for three groups of the healthy populations (Appendix I: Table A4). For each analyte of the healthy populations, the upper limit cut-offs were set at above the 97.5th percentiles, whereas the lower limit cut-offs were set at below the 2.5^{th} percentiles. Table 3.1 presents the cut-off values for the analytes and marker ratios for the screening of aminoacidopathies, organic acidemias, and FAO disorders. Clinical manifestations of the patients, previous family histories, unexplained deaths of siblings, and consanguinity of the parents, etc. had considered for the screening of specific IEM.

Table 3.1: The cut-off values of the analytes and marker ratios for the screening of IEM.

Suspected disease	Analytes/Marker ratios	Cut-offs Group A (µmol/L)	Cut-offs Group B (µmol/L)	Cut-offs Group C (µmol/L)
PKU	↑ Phe	> 85.45	> 88.12	> 82.08
	↑ Phe/tyr	> 3.47	> 5.11	> 8.03
BHD	↑ Phe/tyr	> 3.47	> 5.11	> 8.03
CIT	↑ Cit	> 17.17	> 35.91	> 39.74
	↑ Cit/Phe	> 0.42	> 0.75	> 0.80
TYR	↑ Tyr	> 199.96	> 128.12	> 136.63
	↑ Tyr/Phe	> 5.90	> 2.50	> 2.56
MSUD	↑ Val	> 188.83	> 187.45	> 210.34
	↑ Xle	> 200.85	> 201.74	> 220.69
	↑ Val/Phe	> 5.37	> 3.35	> 4.05
	↑ Xle/Ala	> 1.37	> 1.44	> 1.23
	↑ Xle/Phe	> 5.62	> 3.37	> 4.14

Table 3.1 (continued).

ARG	↑ Arg	> 21.39	> 74.98	> 95.36
	↑ Arg/Orn	> 0.26	> 1.00	> 1.04
OTC	↓ Cit	< 4.68	< 11.51	< 13.67
	↑ Orn	>112.77	> 145.61	> 148.49
HCY	↑ Met	> 42.38	> 40.65	> 42.17
	↑ Met/Phe	> 0.74	> 0.77	> 0.78
MMA	↑ C3	> 6.88	> 2.69	> 2.70
	↑ C3/C2	> 0.19	> 0.15	> 0.14
IVA	↑ C5	> 0.55	> 0.26	> 0.29
	↑ C5/C3	> 0.42	> 0.16	> 0.19
GA-II	↑ C4	> 0.63	> 0.33	> 0.38
	↑ C8	> 0.14	> 0.14	> 0.30
	↑ C14	> 0.51	> 0.27	> 0.13
	↑ C16	> 7.23	> 2.47	> 2.20
	↑ C12	> 0.23	> 0.14	> 0.15
GA-I	↑ C5DC	> 0.29	> 0.17	> 0.20
CUD	↓ C0	< 15.60	< 12.34	< 13.38
	↓ C2	< 6.88	< 8.71	< 9.05
CPT-I	↑ C0	> 73.73	> 69.76	> 75.67
	↑ C0/(C16+C18)	> 21.67	> 43.54	> 56.08
	↓ C16	< 0.88	< 0.61	< 0.55
SCAD	↑ C4	> 0.63	> 0.33	> 0.38
	↑ C4/C2	> 0.03	> 0.02	> 0.02
MCAD	↑ C6	> 0.09	> 0.09	> 0.12
	↑ C8	> 0.14	> 0.14	> 0.30
	↑ C10	> 0.18	> 0.23	> 0.58
	↑ C10:1	> 0.11	> 0.16	> 0.21
	↑ C10:2	> 0.02	> 0.08	> 0.08
	↑ C8/C10	> 1.67	> 4.43	> 2.45
	↑ C8/C2	> 0.01	> 0.01	> 0.02

Table 3.1 (continued).

VLCAD	↑ C14:1	> 0.34	> 0.16	> 0.26
	↑ C14:1/C16	> 0.09	> 0.16	> 0.22
	↑ C14	> 0.51	> 0.27	> 0.13
CPT-II	↑ C16	> 7.23	> 2.47	> 2.20
CACT	↑ C18	> 1.69	> 0.91	> 0.88
	↑ C18:1	> 1.98	> 1.57	> 1.67
TFP	↑ C18OH	> 0.05	> 0.01	> 0.01
	↑ C18:1OH	> 0.03	> 0.02	> 0.02

Abbreviations: PKU, Phenylketonuria; BHD, BH4 deficiency; CIT, citrullinemia ; TYR, Tyrosinemia; MSUD, maple syrup urine disease; ARG, Argininemia; OTC, Ornithine transcarbamylase deficiency; HCY, homocystinuria; MMA, methylmalonic acidemia; IVA, isovaleric acidemia; GA_II, glutaric acidemia type II (multiple acyl-CoA dehydrogenase deficiency); GA-I, glutaric acidemia type I; CUD, carnitine uptake defect; CPT-I, carnitine palmitoyltransferase I deficiency; SCAD, Short-chain acyl-CoA dehydrogenase deficiency; MCAD, medium-chain acyl-CoA dehydrogenase deficiency; VLCAD, very long-chain acyl-CoA dehydrogenase deficiency; CPT-II, carnitine palmitoyltransferase II deficiency; CACT, carnitine-acylcarnitine translocase deficiency; TFP, trifunctional protein deficiency.

3.1.3. Family and clinical history suggestive of inborn errors of metabolism (IEM) among suspected patients with IEM

Among 273 suspected patients with IEM, 132 (48.4%) were children of consanguineous parents, 78 (28.6%) had abnormal birth events, 38 (13.9%) had a history of unexplained deaths of siblings and 40 (14.7%) patients had a positive family history. Most of the patients had more than one clinical manifestations such as seizure (45.8%, N = 125), developmental delay (44.0%, N = 120), lethargy (42.5%, N = 116), irritation (42.1%, N = 115), poor feeding (39.2%, N = 107%), abnormal movement (28.9%, N = 79), vomiting (19.0%, N = 52), tachypnea (8.4%, N =23) and toe walking (6.6%, N = 18) (Appendix I: Table A5). Clinical manifestations of these patients overlapped with each other and most of the patients were presenting at least two of the aforementioned clinical signs and symptoms. After clinical evaluation, blood specimens of these suspected patients with IEM were analyzed using LC-MS/MS for differential diagnosis of IEM.

3.1.4. Results for IEM from clinically suspected patients using LC-MS/MS

Among 273 clinically suspected patients with IEM, nine patients came out as screening positive for six different IEM by LC-MS/MS analysis of DBS cards. Among these nine patients, there were three cases of phenylketonuria (PKU); one case for each of citrullinemia type II (CIT-II), methylmalonic acidemia (MMA), isovaleric acidemia (IVA), and carnitine uptake defect (CUD); and two cases of medium-chain acyl-CoA

dehydrogenase deficiency (MCAD). Two female patients were found to be screening positive for PKU at the age of about 1 year and the only male patient was screening positive at the age of 10 years. The major clinical complications of these PKU patients were developmental delays, lethargy, and seizures (Table 3.2). LC-MS/MS generated chromatogram of phenylalanine level of a PKU patient and a healthy control has been shown in Fig 3.1(a).

The male patient was positive for citrullinemia type II (CIT-II) upon screening at the age of 10 years and his major clinical complications included irritability, restlessness, and excessive crying followed by unconsciousness (Table 3.2). A representative chromatogram of blood citrulline level of a Cit-II diagnosed patient and a healthy subject has been presented in Fig 3.1(b).

A one-year-old boy with clinical manifestations of seizure, low muscle tone, and developmental delay came out as screening positive for methylmalonic acidemia (MMA). Comparison of blood C3 level of MMA patient and healthy control has been shown in Fig 3.1(c).

A male patient came out as screening positive at the age of 1.9 years for isovaleric acidemia (IVA) and the condition was confirmed by urine metabolomic profiling using GC-MS. He was hospitalized multiple times with clinical manifestations like acute respiratory infections and acute watery diarrhea. He had developmental delay and an elevated level of plasma ammonia. IVA diagnosed patient's blood level of C5 has been compared with healthy control in Fig 3.1(d).

A screening positive male patient for carnitine uptake deficiency (CUD) using LC-MS/MS had clinical complications like lethargy, restlessness, poor feeding, seizure, vomiting, abnormal movement, developmental delay, speech problems, and the inability of walking. Fig 1(e) shows the chromatograms for C0 and C2 level.

Table 3.2: Abnormal blood concentrations of metabolites and marker ratios together with clinical manifestations of the patients with suspected IEM, as screened by LC-MS/MS.

Name of Disorders	Total no. of positive cases (frequency among detected cases)	Case ID	Age at diagnosis	Metabolites or marker ratios	Concentrations of metabolites (µmol/L) or marker ratios	Cut-offs (µmol/L)	Major clinical complications
PKU	3 (33.3%)	Case 1	1.1 years	Phe	180.12	> 88.12	Lethargy, irritation, Seizure and developmental delay
				Phe/tyr	5.97	> 5.11	
		Case 2	1.0 year	Phe	282.45	> 88.12	
				Phe/tyr	6.30	> 5.11	
		Case 3	10.0 years	Phe	1170.32	> 82.08	
				Phe/tyr	46.58	> 8.03	
CIT-II	1 (11.1%)	Case 4	10.0 years	Cit	1494.66	> 39.74	Irritability, restlessness and excessive crying followed by unconsciousnes s
				Cit/Phe	26.68	> 0.80	
MMA	1 (11.1%)	Case 5	1.0 year	C3	5.39	> 2.69	Developmental delay, seizure and low muscle tone
				C3/C2	0.29	> 0.15	
IVA	1 (11.1%)	Case 6	1.9 years	C5	13.80	> 0.26	Recurrent infection and developmental delay
				C5/C3	25.07	> 0.16	
CUD	1 (11.1%)	Case 7	2.0 years	C0	6.13	< 12.34	Lethargy, restless, poor feeding, seizure, vomiting, developmental delay, speech problem and the inability of walking
				C2	6.47	< 8.71	

Table 3.2 (continued).

MCAD	2 (22.2%)	Case 8	10.0 years	C6	0.62	> 0.12	Restlessness, irritability, abnormal behavior, speech problem and mental retardation
				C8	1.90	> 0.30	
				C10	2.90	> 0.58	
				C10:1	1.14	> 0.21	
				C10:2	0.04	> 0.08	
				C8/C10	0.65	> 2.45	
				C8/C2	0.16	> 0.02	
		Case 9	8.5 years	C6	0.23	> 0.12	
				C8	1.15	> 0.30	
				C10	1.88	> 0.58	
				C10:1	0.66	> 0.21	
				C10:2	0.03	> 0.08	
				C8/C10	0.61	> 2.45	
				C8/C2	0.09	> 0.02	

Abbreviations: PKU, phenylketonuria; CIT-II, citrullinemia type II; MMA, methylmalonic acidemia; IVA, isovaleric acidemia; CUD, carnitine uptake defect; and MCAD, medium-chain acyl-CoA dehydrogenase deficiency.

Two patients, a male and a female who were screening positive for medium-chain acyl-CoA dehydrogenase deficiency (MCAD), were siblings. At the time of screening using LC-MS/MS, the male patient was 10 years old, whereas the female patient was 8.5 years old. Irritability, restlessness, abnormal behavior, mental retardation, and speech problem were common clinical manifestations of these patients. The concentrations of metabolites in the blood (C6, C8, C10, C10:1) and C8/C2 ratio of both participants were slightly higher than that of the cut-off values, while the concentration of C10:2 and the C8/C10 ratio were within the reference cut-off values in case of both male and female patients (Table 3.2). A representative chromatogram of the analytes of screening positive MCAD patient and healthy control has been presented in Fig 3.1 (f).

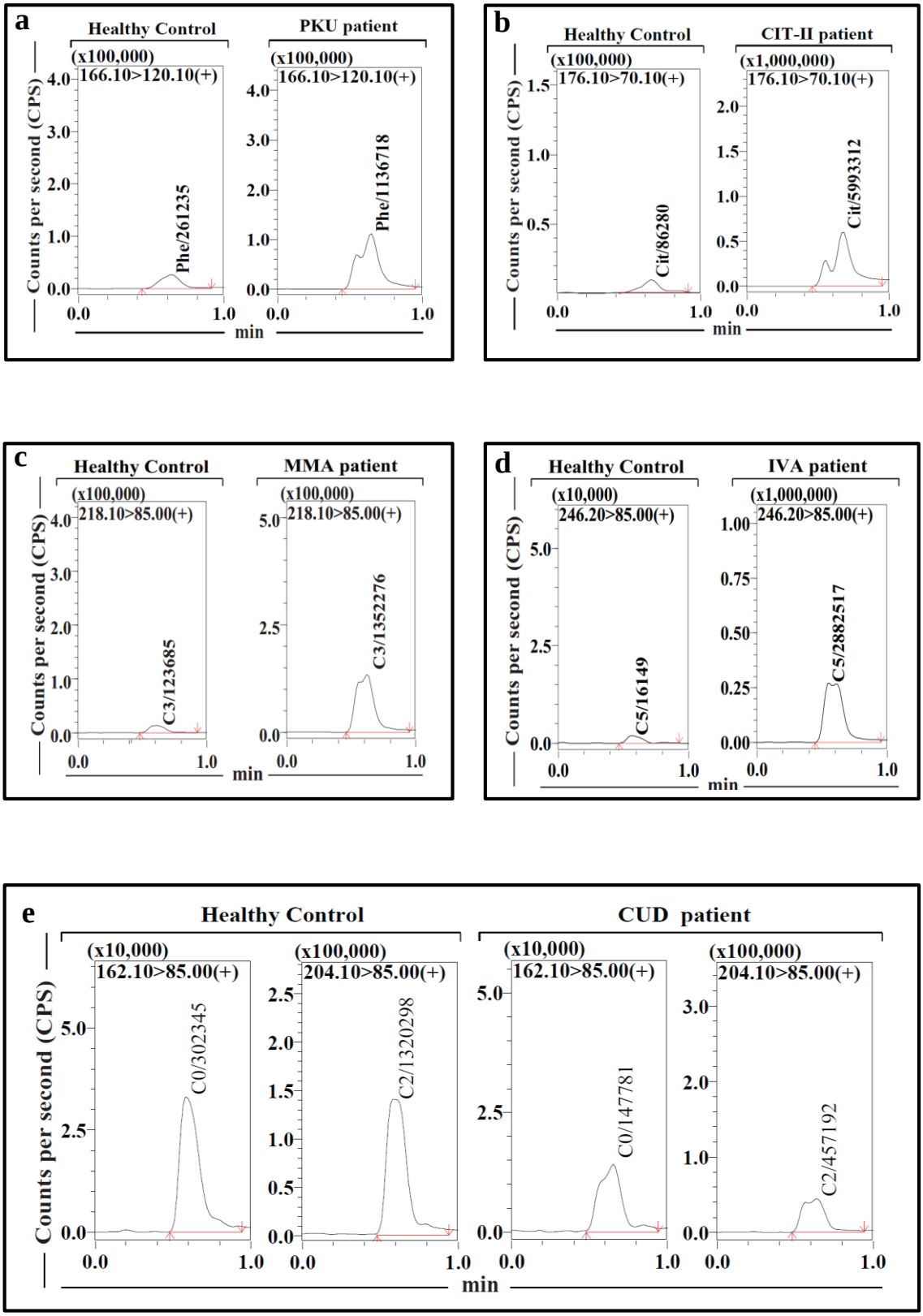
a
Healthy Control
PKU patient
(x100,000)
166.10>120.10(+)
Phe/261235
Phe/1136718
b
Healthy Control
CIT-II patient
(x100,000)
(x1,000,000)
176.10>70.10(+)
Cit/86280
Cit/5993312
c
Healthy Control
MMA patient
(x100,000)
218.10>85.00(+)
C3/123685
C3/1352276
d
Healthy Control
IVA patient
(x10,000)
(x1,000,000)
246.20>85.00(+)
C5/16149
C5/2882517
e
Healthy Control
CUD patient
(x10,000)
(x100,000)
162.10>85.00(+)
204.10>85.00(+)
C0/302345
C2/1320298
C0/147781
C2/457192
Counts per second (CPS)
min

Fig 3.1 (continued).

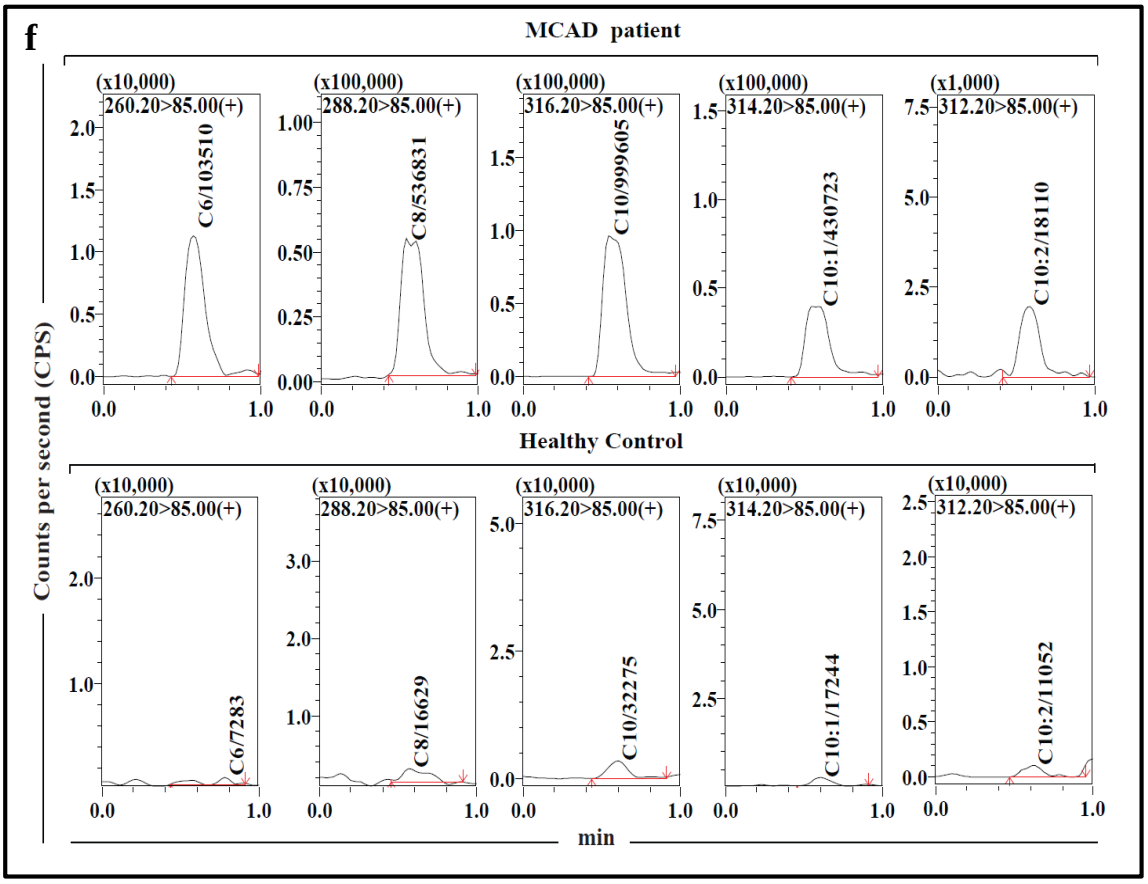

Fig 3.1: Comparison of representative chromatograms of analytes between Phenylketonuria (PKU), Citrullinemia type II (CIT-II), Isovaleric acidemia (IVA), Methylmalonic acidemia (MMA), Carnitine uptake defect (CUD), Medium-chain acyl-CoA dehydrogenase deficiency (MCAD) patients and healthy participants. The X-axis represents the time after the run in minute and Y-axis represents metabolites counted per second (CPS). The upper-left number inside the MRM chromatogram represents precursor ion followed by > sign and then the upper right number represents the target ion. Each metabolite detected has been shown vertically above the peak with a capital C followed by digit(s) and then IS for the internal standard. The concentration of each metabolite is proportional to the peak area. LC-MS/MS chromatograms of patients with IEM and healthy control has been presented as (a) Blood phenylalanine level of healthy participant and PKU patient, (b) Blood citrulline level of healthy participant and CIT-II patient, (c) Blood C3 (propionylcarnitine) level of healthy participant and MMA patient, (d) Blood C5 (isovaleryl-/2-methylbutyryl carnitine) level of healthy participant and IVA patient , (e) Blood C0 (free carnitine) and C2 (acetylcarnitine) level of healthy participant and patient, (f) Blood C6 (hexanoylcarnitine), (C8) octanoylcarnitine, C10 (decanoylcarnitine), 10:1 (decenoylcarnitine) and C10:2 (decadienoylcarnitine) level of healthy participant and patient.

Abbreviations: PKU: Phenylketonuria; CIT-II, Citrullinemia type II; MMA, Methylmalonic acidemia; IVA, Isovaleric acidemia; CUD, Carnitine uptake defect and MCAD, Medium-chain acyl-CoA dehydrogenase deficiency.

3.1.5 GC-MS-based second-tier test for analysis of urine specimens from the LC-MS/MS-based screening positive IEM cases

LC-MS/MS analysis revealed that the blood levels of amino acids and acylcarnitines were abnormal in specimens of 9 patients. Next, urine specimens from all these nine patients were subjected to a second-tier test using GC-MS. The urinary metabolic profiling of three screening positive patients with PKU revealed elevated levels of 4-hydroxyphenylacetic acid, phenyllactic acid, 4-hydroxyphenyllactic acid, 2- hydroxyphenylacetic acid and mandelic acid. Elevated levels of these organic acids are suggestive of PKU caused by the deficiency of phenylalanine hydroxylase enzyme (Appendix I: Table A6).

In the case of CIT-II screening positive patient, urinary metabolite profiling using GC-MS revealed an elevated level of citrulline, which is suggestive of citrullinemia (Appendix I: Table A6). The GC-MS analysis of urine specimens from screening positive patient with MMA showed an elevated level of urinary excretion of methylmalonic acid, suggesting the confirmatory diagnosis of the screening positive MMA case (Appendix I: Table A6). The second-tier test using GC-MS analysis of urine specimen from IVA screening positive case revealed that IVA specific metabolites such as isovalerylglycine and 3-OH-isovalerate were elevated (Appendix I: Table A6).

The urine metabolic profiling of screening positive patient with CUD revealed elevated levels of urinary excretions of 3-hydroxy butyric acid (3HB), adipic acid, and p-cresol. An elevated level of adipic acid along with a highly elevated level of 3HB is suggestive of carnitine uptake deficiency (Appendix I: Table A6).

For the MCAD screening positive patients which had LC-MS/MS-derived borderline positive values above the cut-off, urinary metabolic profiling by GC-MS revealed that the levels of all the analytes tested were within the range of the cut-off, which confirms that these patients were not true cases of MCAD, i.e., false positive (Appendix I: Table A6).

3.2. Molecular analysis of glucose-6-phosphate dehydrogenase (G6PD) gene among Bangladeshi individuals

3.2.1 Cut-off value determination for G6PD deficiency in the study population

The study enrolled a total of 121 (male: 79 and female: 42) clinically suspected participants. Table 3.3 shows the G6PD enzyme activity profile for the study participants. Five male participants who had less than 10% of the median value of G6PD enzyme activity for all male participants were excluded to calculate the adjusted median G6PD enzyme activity.

The study population had a median G6PD activity of 12.73 U/g Hb (range: 0.7–24.57 U/g Hb). On the other hand, according to Domingo *et al.*, the adjusted male median enzyme activity (12.28 U/g Hb) was considered as 100% (Fig 3.2) [78]. The participants with enzyme activity less than 7.37 U/g Hb (60% of adjusted male median) were classified as deficient. Finally, 14 out of 121 study participants (12 males and 2 females) had been suspected as G6PD deficient based on this cut-off value.

Table 3.3: Reference values to describe the G6PD activity profile of the study population.

Reference values	Total Participants (N = 121) (U/g Hb)	Female (N = 42) (U/g Hb)	Male (N = 79) (U/g Hb)	Adjusted male (N = 74) (U/g Hb)
Mean	12.49	13.59	11.90	12.64
Standard deviation	5.34	4.37	5.73	5.12
Median	12.73	13.78	12.02	12.28
Range	0.7–24.57	3.06–24.57	0.7–23.72	1.38–23.72

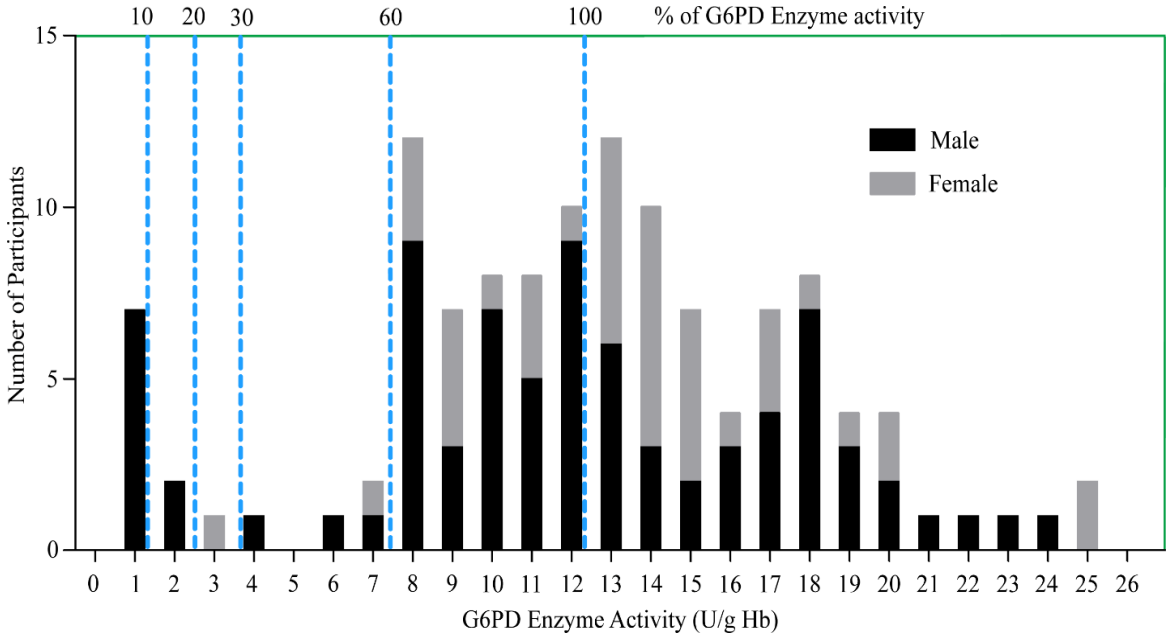

Fig 3.2: Proportion of G6PD enzyme activity levels for male and female participants compared to the adjusted male median value. For each G6PD enzyme activity level (U/g Hb) shown in the X-axis, the corresponding value in the Y-axis indicates the number of participants. The numbers on top of each dotted line, shown as 10, 20, 30, and 60 on the uppermost horizontal line of the graph indicate different cut-off values as percentages for the study population. 60% (shown as 60) of the adjusted male median (shown as 100) is the upper limit of cut-off value and the participants with enzyme activities below 60% are considered deficient. The black portion of each bar indicates the male participants, whereas gray portion of each bar indicates the female participants.

3.2.2 Pinpointing G6PD mutations in deficient participants

Mutation detection by Sanger sequencing revealed that there were three different single missense mutations (c.C131G, c.G487A, and c.G949A) in the G6PD gene of twelve G6PD-deficient specimens. Among these 12 G6PD-deficient specimens, six had c.C131G, five had c.G487A and one had c.G949A mutation(s). According to the database (http://www.bioinf.org.uk/g6pd/), all these three mutations are already known variants, named as G6PD Orissa (c.C131G or Ala44Gly), G6PD Mahidol (c.G487A or Gly163Ser), and G6PD Kalyan-Kerala (c.G949A or Glu317Lys). A representative NCBI BLAST result and Sanger's sequencing chromatogram of G6PD gene sequence containing G6PD Orissa, G6PD Mahidol, and G6PD Kalyan-Kerala has been shown in Fig 3.3, 3.4 and 3.5, respectively. Blast results and sequencing chromatogram clearly demonstrate the single nucleotide mutation in the G6PD gene of the deficient patients. It should be mentioned here that two (one male and one female) suspected G6PD deficient participants were excluded from the actual G6PD deficient category because there were no mutations found in the G6PD gene of these patients.

These anomalies could be explained by the enzyme activities (57.16% and 58.43% of the adjusted male median value) of those two specimens which were insignificantly lower than the cut-off value, which was 60% of adjusted male median. Thus these two specimens can be regarded as false positive as proposed by Domingo *et al.* [78]. The, results, therefore, suggest that the combined results of FST, G6PD enzyme activity assay and gene sequencing could accurately inform whether a participant is G6PD-deficient. Accordingly, although FST and G6PD enzyme activity assays could identify 14 participants as G6PD deficient, G6PD gene sequencing results excluded two participants from the deficient category. Thus, there were 12 participants with actual G6PD deficiency, suggesting that the frequency of G6PD deficiency among the study population was 9.9%. The male participants showed a frequency of 13.9%, whereas female participants showed 2.4% frequency.

Fig 3.3: A representative NCBI BLAST result of G6PD gene sequence and Sanger's sequencing chromatogram of G6PD Orissa (c.C131G or Ala44Gly) mutation. (a) BLAST result of the G6PD gene sequence containing Orissa mutation showing C to G mutation. The nucleotide marked as a red bar indicates the mismatch position with the wild type gene of G6PD mRNA sequence (transcript variant 2). (b) Partial Sanger's sequencing chromatogram of G6PD gene sequencing containing G6PD Orissa mutation. The nucleotide marked as red bar indicates the position of C to G substitution.

Fig 3.4: A representative BLAST result and sequencing chromatogram of G6PD Mahidol mutation (c.G487A or Gly163Ser). (a) BLAST result of the G6PD gene sequence containing Mahidol mutation showing G to A substitution. The nucleotide marked as a red bar indicates the mismatch position with the wild type gene of G6PD mRNA sequence (transcript variant 2). (b) Partial Sanger's sequencing chromatogram of G6PD gene sequencing containing G6PD Mahidol mutation. The nucleotide marked as red bar indicates the position of G to A substitution.

Fig 3.5: A representative blast result of G6PD Kalyan-Kerela (c.G949A or Glu317Lys) mutation and partial chromatogram of Sanger's sequencing. (a) BLAST result of the G6PD gene sequence containing Kalyan-Kerela mutation showing G to A mutation. The nucleotide marked as a red bar indicates the mismatch position with wild type gene of G6PD mRNA sequence (transcript variant 2). (b) Partial Sanger's sequencing chromatogram of G6PD gene sequencing containing G6PD Kalyan-Kerela mutation. The nucleotide marked as red bar indicates the position of G to A substitution.

3.2.3 Comparison of biochemical parameters between deficient and non-deficient individuals in the G6PD molecular component of the study

G6PD deficiency mostly affects RBC and this disorder can be life-threatening under conditions of oxidative stress which affects RBC parameters like Hb, reticulocyte, etc. The level of Hb concentrations and reticulocytes counts were compared between G6PD deficient and non-deficient participants to check whether these parameters varied between these two groups of participants. The mean (± SD) Hb concentration of 109 G6PD non-deficient participants was higher (16.29 ± 3.16 g/dL, range: 6.5 g/dL -22.0 g/dL) than that of 12 G6PD deficient patients who had average hemoglobin level 14.84 ± 2.87 g/dL (mean ± SD), ranging from 10 g/dL to 19 g/dL (Table 3.4). As shown in Fig 3.6, there was no significant difference in hemoglobin levels between participants with or without G6PD deficiency ($p > 0.05$). The reticulocyte counts were in the range of 0.4–3.2% for the study population (Table 3.4). The proportional range of reticulocytes is considered normal for ages between 3 months to 18 years [88] and the participants of this study fall within this age range. There were no significant differences in reticulocyte counts between deficient (mean ± SD: 1.41 ± 0.73%) and non-deficient (mean ± SD: 1.44 ± 0.56) participants ($p > 0.05$) (Fig 3.6). Thus it can be concluded that when G6PD deficient individuals remain in a state of remission, their Hb levels, and reticulocyte counts do not vary significantly from non-deficient individuals.

Table 3.4: Distribution of demographic information between G6PD-nondeficient and G6PD-deficient participants.

Participants	Number	Hb range (g/dL)	Average Hb (mean ± SD)	Reticuloctyes (% of RBCs)	Average Reticulocytes (mean ± SD)
Non-deficient	109	6.5–22.0	16.29 ± 3.16	0.4–3.2	1.44 ± 0.56
Deficient	12	10–19.0	14.84 ± 2.87	0.5–2.9	1.41 ± 0.73

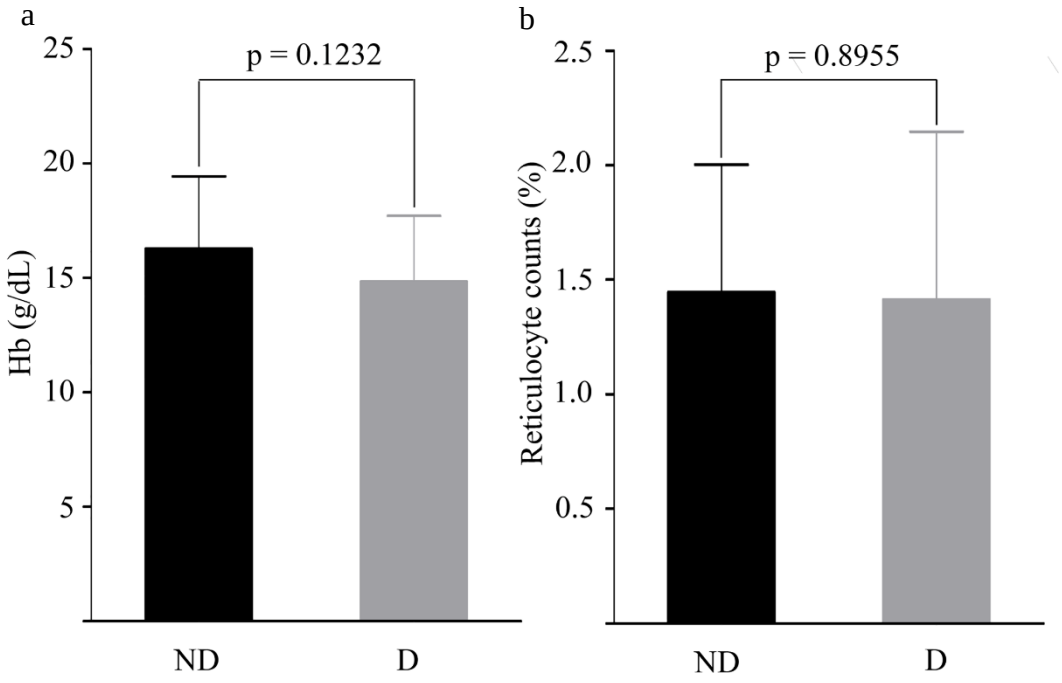

Fig 3.6: Hemoglobin levels and reticulocyte counts in the G6PD-non-deficient (ND) and deficient (D) participants. (a) Depicts hemoglobin levels (g/dL), and (b) Demonstrates reticulocyte counts (%) in non-deficient and deficient participants. A p-value < 0.05 was considered statistically significant.

3.2.4 G6PD gene mutations and enzyme activity levels

The study demonstrates that enzyme activity of 5 male participants identified with Orissa mutation ranged from 0.7 U/g Hb to 1.68 U/g Hb and this range corresponded to 5.7%-13.68% of normal G6PD activities (Table 3.5) provided that the adjusted male median 12.28 U/g Hb was considered 100%. The mean enzyme activity of G6PD Orissa male subjects was (1.194 ± 0.37) U/g Hb (mean ± SD) which was (9.72 ± 3.01) % of adjusted male median. Moreover, a female was identified with homozygous Orissa mutation with an enzyme activity of 3.06 U/g Hb (24.92% of adjusted male median) which was higher than that of her male counterparts and this discrepancy can be attributed to higher reticulocyte counts (2.9%) in this female infant compared to the relatively lower range of reticulocyte counts (0.5-1.6%) in G6PD-deficient Orissa males (Table 3.5), supporting that the G6PD enzyme activity of a reticulocyte is higher than that of a mature erythrocyte [76]. The substitution of alanine by glycine at the 44^{th} position observed in the Orissa mutation causes an increase in K_m for $NADP^+$ at the cofactor binding site, which in turn results in a reduction in G6PD enzyme activity level [59].

Table 3.5: Fluorescent spot test and enzyme assay results of G6PD-deficient samples along with corresponding WHO classification.

Sample	FST result	Enzyme activity (U/g Hb)	Percent of normal enzyme activity	WHO classification (enzyme activity)
Orissa-1	No fluorescence	0.70	10.04%	Class III (10-60%)
Orissa-2	No fluorescence	1.03	14.77%	Class III (10-60%)
Orissa-3	Slight fluorescence	1.17	16.78%	Class III (10-60%)
Orissa-4	Slight fluorescence	1.39	19.94%	Class III (10-60%)
Orissa-5	No fluorescence	1.68	24.10%	Class III (10-60%)
Orissa-6	Moderate fluorescence	3.06	43.90%	Class III (10-60%)
Mahidol-1	No fluorescence	0.75	10.76%	Class III (10-60%)
Mahidol-2	No fluorescence	1.04	14.62%	Class III (10-60%)
Mahidol-3	No fluorescence	1.43	20.51%	Class III (10-60%)
Mahidol-4	No fluorescence	1.63	23.38%	Class III (10-60%)
Mahidol-5	Moderate fluorescence	3.58	51.36%	Class III (10-60%)
Kalyan-Kerala-1	Normal pattern of fluorescence	6.41	92%	Class IV (60-150%)

G6PD Mahidol (c.G487A or Gly163Ser) mutation was found in 5 male subjects with G6PD enzyme activity 6.10%-29.15% of the adjusted male median value; 0.75 U/g Hb being the lowest enzyme activity and 3.58 U/g Hb being the highest. The mean enzyme activity of

G6PD Mahidol subjects was 1.68 ± 1.11 U/g Hb or 13.68 ± 9.04% (mean ± SD) of the adjusted male median. The difference in G6PD activity level among G6PD Mahidol participants might be due to differences in reticulocytes counts (0.7–2.6%) because a reticulocyte may have as much as 5-fold higher G6PD enzyme activity than a fully matured erythrocyte [76]. The substitution of glycine by serine at 163rd position in Mahidol variant affects G6PD protein structure and stability, but not the catalytic efficiency of G6PD enzyme [89, 90].

A male participant was identified with G6PD Kalyan-Kerala (G949A or GLU317Lys) mutation. The G6PD enzyme activity of this Kalyan-Kerala mutant subject was found to be much higher (52.2%) than that of the Kalyan-Kerala mutant subjects showing 20% G6PD enzyme activity of normal subjects in a study by Kaeda *et al.* [59]. On a retrospective investigation, it was learned that the patient had previously undergone blood transfusion, which may account for the enhanced G6PD enzyme activity. Substitution of an acidic glutamate residue by a basic lysine residue in Kalyan-Kerala mutants disrupts the structure of the G6PD enzyme which causes a decrease in electrophoretic mobility compared to the wild type G6PD enzyme [91].

3.3 Establishment of high-resolution melting (HRM) curve analysis based method for detection of G6PD variants in heterozygous females

3.3.1 Demographic information and G6PD enzyme status of the study participants

Depending on the history of the previous hemolytic crisis, all 63 Bangladeshi Bengali female participants who were in the age range of 0–15 years were suspected of having G6PD deficiency. The mean age of the study participants was 8.47 ± 3.24 years. Diagnosis of actual G6PD deficiency was performed by analyzing blood specimens using the quantitative enzyme assay method. Among 63 participants, only 4 (6.34%) participants were detected as G6PD enzyme deficient, provided that the cut-off for G6PD enzyme deficiency was set to 7.37 U/g Hb which has been established in this study [81].

3.3.2 HRM-based assessment of samples targeting the common G6PD variants in Bangladesh

All 63 specimens were subjected to HRM analysis targeting three G6PD common variants in Bangladesh which were identified from this study. HRM curve analysis could identify 7 out of 63 samples that were found to vary from the wild-type alleles (Figure 3.7a, 3.7b, and 3.7c). The results of HRM curve analysis were confirmed by Sanger's DNA sequencing.

There were no G6PD variants or SNPs in the specimens that formed cluster with the wild type control samples.

Three out of seven samples that had deviated from wild type allele were found to have the enzyme activities lower than the cut-off (3.1 U/g Hb, 4.89 U/g Hb, and 5.45 U/g Hb), whereas the enzyme activities of the remaining four samples were greater than that of the cutoff (7.67 U/g Hb, 9.38 U/g Hb, 10.84 U/g Hb, and 11.34 U/g Hb) (Table 3.6). A specimen with G6PD Orissa variant in a homozygous state had an enzyme activity of 3.1 U/g Hb, whereas the same variant was found in one of the seven samples in a heterozygous state with an enzyme activity of 4.89 U/g Hb (Table 3.6). The sample with an enzyme activity of 5.45 U/g Hb was found to have G6PD Kalyan-Kerala (c.G949A) variant in a heterozygous state (Table 3.6).

Table 3.6: G6PD enzyme activity in the samples with G6PD Orissa, G6PD Mahidol, and G6PD Kalyan-Kerala variants.

Mutations	Exons	Amino acid substitution	Enzyme activity (U/g Hb)	Enzyme activity compared to the cut-off (%)
c.C131G*	Exon 3	p. Ala44Gly	3.10	57.93% ↓
c.C131G#	Exon 3	p. Ala44Gly	4.89	33.60% ↓
c.G487A#	Exon 6	p. Gly163Ser	7.67	03.91% ↑
c.G487A#	Exon 6	p. Gly163Ser	11.34	35.04% ↑
c.G949A#	Exon 9	p. Glu317Lys	5.45	26.03% ↓
c.G949A#	Exon 9	p. Glu317Lys	9.38	21.45% ↑
c.G949A#	Exon 9	p. Glu317Lys	10.84	33.99% ↑

#indicates heterozygous variants; *indicates homozygous variants; ↓ indicates lower; ↑ indicates higher

Sequencing results also confirmed that four samples with enzyme activities greater than the cut-off but varied from the wild-type samples had genetic polymorphisms. All of these four specimens had heterozygous G6PD variants. The enzyme activities of two of these specimens with heterozygous G6PD Mahidol (c.G487A) variant were 7.67 U/g Hb and 11.34 U/g Hb, respectively, whereas the other two specimens with G6PD Kalyan-Kerala heterozygous variant had enzyme activities of 9.38 U/g Hb and 10.84 U/g Hb, respectively. Overall, these data suggest that HRM curve analysis targeting the common G6PD variants

can be successfully applied for screening of G6PD variants in Bangladesh with 100% sensitivity and 100% specificity.

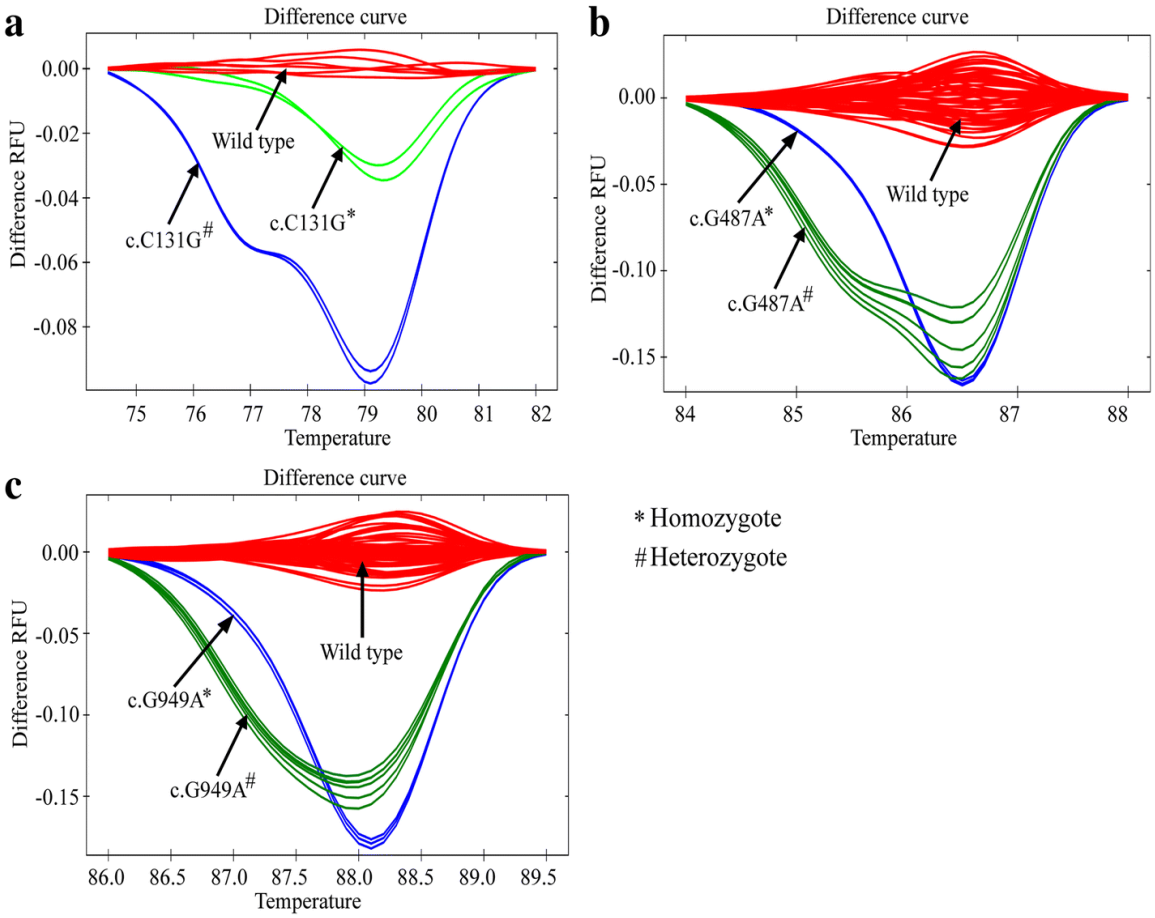

Fig 3.7: HRM curves patterns for the indicated common mutations in the glucose-6-phosphate dehydrogenase gene for the study participants to differentiate homozygous and heterozygous states from each other and from the wild-type alleles. (a) G6PD Orissa mutation, (b) G6PD Mahidol mutation and (c) G6PD Kalyan-Kerala mutation in homozygous or heterozygous states which could be unambiguously distinguished from the wild-type alleles.

3.3.3 HRM-based assessment of samples targeting the G6PD variants other than common mutations

When all 63 samples were subjected to HRM analysis to screen other unreported variants, only two samples were found to vary from the wild-type alleles (Fig 3.8). DNA sequencing revealed that both of these two specimens had G6PD Mediterranean variant (c.C563T) in heterozygous states and their enzyme activities were 3.52 U/g Hb and 11.89 U/g Hb (Table 3.7), respectively. There were no G6PD variants in the specimens when they formed melt curve clusters with the wild type reference specimens, suggesting 100% sensitivity and specificity of the HRM-method. Thus, similar to other G6PD common variants, HRM can

also target G6PD Mediterranean variants to detect G6PD heterozygote states among Bangladeshi individuals.

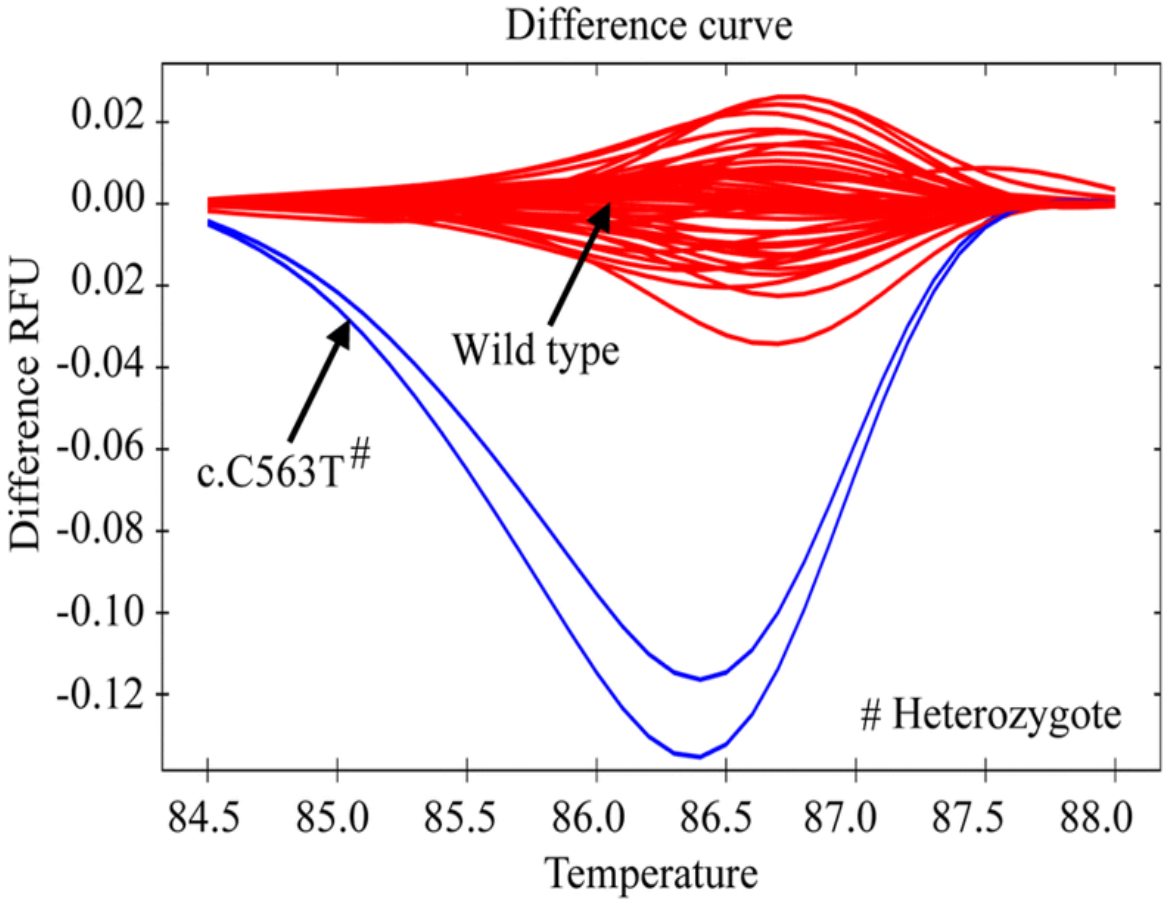

Fig 3.8: Identification of G6PD Mediterranean (c.C563T) mutations in a heterozygous state by using primers targeting exon-6. The HRM curve pattern for c.C563T heterozygous mutation could be distinguished from the wild-type allele.

3.3.4 Skewing of G6PD enzyme activities in heterozygous female participants

The results described so far demonstrated that 8 out of 63 female samples had heterozygous variants. Next, the investigation was done to reveal the variation of the G6PD enzyme activity level among the samples with different heterozygous states and within different samples of the same heterozygous states. Tables 3.6 and 3.7 show that samples with heterozygous variants had different levels of G6PD enzyme activities. Even samples with the same heterozygous variants differed greatly among one another in terms of the levels of G6PD enzyme activities. One out of eight samples with the heterozygous state had c.C131G variant and its enzyme activity was 4.89 U/g Hb, which was 33.60% lower than the cut-off 7.37 U/g Hb (Table 3.6). However, two of the specimens with c.G487A heterozygous variant had enzyme activities of 7.67 U/g Hb and 11.34 U/g Hb, which were

03.91% and 35.04% higher than the cut-off value (Table 3.6), respectively. Two samples with heterozygous c.C563T variants showed significant differences in their enzyme activities and the results were comparable to the c.G487A heterozygous variant. One of these two samples had an enzyme activity of 3.52 U/g Hb, which was 52.19% lower than the cut-off, whereas the other one had 38.02% higher enzyme activity than the cut-off (Table 3.7). Finally, 3 samples with c.G949A heterozygous state were identified in the study. One of these three samples had an enzyme activity of 5.45 U/g Hb, which was 26.03% lower than the cut-off. However, the rest two specimens with c.G949A heterozygous variant had enzyme activities of 9.38 U/g Hb and 10.83 U/g Hb, which were 21.45% and 33.99% higher than the cut-off value (Table 3.6), respectively.

Table 3.7: G6PD enzyme activities in the samples with heterozygous G6PD Mediterranean variants.

Mutations	Exons	Amino acid substitution	Enzyme activity U/g Hb	Enzyme activity compared to cut-off (%)
c.C563T[#]	Exon 6	p. Ser188Phe	3.52	52.19% ↓
c.C563T[#]	Exon 6	p. Ser188Phe	11.89	38.02% ↑

[#]indicates heterozygous variants; ↓ indicates lower; ↑ indicates higher

DISCUSSION

CHAPTER 4

4. Discussion

4.1. Determination of cut-off values of amino acids and acylcarnitines for screening for IEM using LC-MS/MS in Bangladesh

The number, intricacy, and diverse clinical spectrum of IEM present a daunting diagnostic challenge to the physicians. However, in order to reduce morbidity and mortality, or other severe repercussions like irreversible neurological damage to the patients with IEM, early diagnosis, and the institution of appropriate therapy are very critical. The use of LC-MS/MS during the past decades has led to a remarkable increase in the screening of IEM. Many countries have established newborn screening (NBS) tests using LC-MS/MS which analyzes metabolites from dried blood spots (DBSs) to detect the IEM-associated disorders, particularly the treatable one [6, 92]. Although NBS is not in practice in Bangladesh, the government health policymakers have initiated official processes to start a nationwide NBS program for the management of IEM. Under the circumstances, the establishment of local cut-offs for IEM-associated metabolites is timely. This is the first study on screening of IEM using LC-MS/MS in Bangladesh, where the cut-off values of individual amino acids and acylcarnitines were established by analyzing the DBS specimens of healthy subjects and the patients with suspected IEM were screened using the established cut-off values.

LC-MS/MS has been widely adopted for IEM screening as it offers simultaneous and robust multiple disease screening using a single analytical high throughput technique [21]. Moreover, LC-MS/MS-based screening of IEM provides the advantages of rapidity and convenience in sample collection and the stable isotopic internal standards used for quantification in this method increases the specificity and sensitivity of the test. Furthermore, this cost-effective technique could be used to detect a number of diseases including very rare and common diseases. To reduce the cost, usually, a large number of specimens are analyzed in a single run.

Most aminoacidopathies, organic acidemias, and FAO disorders can be diagnosed using LC-MS/MS with 99% sensitivity and 99.995% specificity [5, 93-95] but it requires the establishment of rigorous reference cut-off limits to detect the IEM-related disorders. Reliable cut-offs would help to minimize false positive or false negative cases [96-98]. Through a worldwide collaborative project, the cut-off values for the screening of IEM were determined using 25-30 million healthy newborns, where 10742 cases were diagnosed with IEM [99]. However, the cut-off limits of metabolites depend on different factors, such

as analysis method, instrument platform, genetic background or ethnicity of a particular population [96, 100]. Since an NBS program does not yet exist in Bangladesh, the patients are hospitalized or they visit the physicians with clinical manifestations of IEM during the postneonatal period when irreversible damage has already occurred. Moreover, clinical manifestations of IEM patients are very puzzling and Bangladesh lacks diagnostic facilities for these disorders. As such, specimens are being sent overseas and the obtained results are interpreted using the cut-off values for other ethnic backgrounds or geographical regions, which may end up in false-positive or false-negative results.

This study is an initiative to overcome those issues, as here, cut-off values for different amino acids and acylcarnitines for three different age groups has been established. Sex-dependent variations of blood amino acids and acylcarnitines concentrations are very rare and this is why only age-specific data has been generated considering that age-specific variations of blood metabolites in the healthy population are frequently reported [101-104]. Moreover, the cut-off limits for the screening of some amino acids and acylcarnitines are very close to normal reference intervals [103].

In this study, all 7 patients diagnosed with IEM were more than one-year-old and they were successfully diagnosed using the established cut-off values for group B (8 days-7 years) and group C (8-17 years), which further emphasizes on the importance of age-specific cut-off values of amino acids and acylcarnitines for diagnosis of IEM. In the study, among 273 patients with clinical signs and symptoms of IEM, 7 (2.6%) patients were diagnosed with IEM using LC-MS/MS analysis from DBS specimens and urinary metabolite profiling using GC-MS.

Different research groups across the world reported different frequencies of IEM; e.g., Han and coworkers confirmed 1135 (6.2%) cases with IEM among 18303 suspected inherited metabolic diseases in China [30], while Al Riyami S *et al.* reported a rate of 10.8% IEM from Oman [105]. Furthermore, frequencies of 0.29%, 0.92%, and 6% IEM cases were reported from Korea, Turkey, and Egypt, respectively [106-108]. The results of this study are not directly comparable with other published survey data because the prevalence rates of IEM vary due to geographical location, ethnicity, instrumentation platform, diagnostic strategies and time span of the surveillance in a particular population [107]. However, despite the difference in criteria for selection of IEM-suspected patients and diagnostic approach, the results of this study are comparable with the published data from India, the

neighboring country of Bangladesh, where IEM frequency has been reported to be as high as 3.2% (113 cases among 3550 suspected patients) [109]. Although the results of this study, which were generated in a time span of two years, do not reflect the true prevalence of IEM in Bangladesh, it clearly demonstrates that IEM are not uncommon in this country and thus the authorities in the health sector and policymakers should be notified of the importance of screening of IEM. In addition, consanguineous marriage, which is a common practice in Muslim countries like Bangladesh, is a major factor behind high rates of IEM [110, 111]. In this study, about 48.4% of the confirmed IEM cases came from consanguineous family history and the findings are consistent with other reports published across the globe [6].

Out of 7 patients who had been diagnosed with IEM using the established cut-off ranges, there were 3 patients with PKU (aminoacidopathies) and 1 patient for each of methylmalonic acidemia and isovaleric acidemia (organic academia), citrullinemia (urea cycle disorder) and carnitine uptake deficiency (FAO). In this study, aminoacidopathies has been identified with higher frequencies than other types of IEM, and a similar pattern was also reported from two other neighboring countries, namely China and India, with PKU as the most prevalent IEM [109, 112].

All 7 patients with IEM disorders that had been diagnosed in our study were treatable. These patients could have a better quality of life and their permanent neurological damage and developmental delay could be prevented provided that they could receive timely treatment after diagnosis through the NBS program. Thus, the implementation of a nationwide newborn screening program to initiate treatment of IEM can prevent the disease severity and permanent mental disability of IEM patients, which ultimately could provide diagnosed children a tolerable good quality of life.

Moreover, the benefits of such a program include societal, ethical, and economic aspects, as the present health expenditures on handicapped people in Bangladesh are huge and beyond the affordable limit for most people. Furthermore, the LC-MS/MS technology has been cost-effective for NBS in many developed countries as well as in developing countries [113-115]. Bangladesh's health policymakers must, therefore, consider LC-MS/MS technology for IEM screening, at least for the most common disorders.

For the diagnosis of IEM, the interpretation of LC-MS/MS results may be inconvenient or arguable if there is a debate about the appropriateness of the reference ranges. In this study, there were two false-positive MCAD cases who had borderline positive concentrations of C6 acylcarnitines on LC-MS/MS analysis and these cases came out as negative when urinary GC-MS analysis was done. The reason for such false-positive cases might be due to the relatively low number of sample size for the determination of cut-offs and this might be seen as the shortfall of the study. More reliable cut-off values are expected to be generated after analyzing more specimens in the future. Additionally, the second-tier tests such as HPLC or GC-MS should be performed by experienced biochemical laboratories and genetic experts.

Usually, various biochemical tests, GC-MS, HPLC, enzymatic assay and molecular analysis can be used as a second-tier or confirmatory test. However, none of these tests offers the conveniences of LC-MS/MS which is why LC-MS/MS has been widely granted and used in the NBS program all across the globe.

Finally, LC-MS/MS techniques may play a vital role in screening and diagnosis of IEM in newborns and this may be helpful in facilitating the timely therapy of treatable IEM. Furthermore, diagnosis of the relatively prevalent metabolic disorders among the Bangladeshi population, along with their clinical features and ages of onsets, may provide physicians with a deeper understanding of IEM, thereby allowing for early diagnosis and treatment initiation. Lastly, due to economic constraints and the high birth rate in the least Developed Countries (LDCs) like Bangladesh, the initiation of the NBS program would be considerable but a worthwhile challenge.

4.2 Molecular analysis of G6PD gene mutations in Bangladesh

The second objective of the study was to reveal the G6PD gene mutations associated with G6PD enzyme deficiency in clinically suspected patients (age 0 to 15 years) in Dhaka, Bangladesh. The patients who had been suspected as G6PD-deficient by both the fluorescent spot test and quantitative enzyme assay were used to identify the underlying mutations. Thus, the combination of fluorescence spot test, enzyme assay, and mutation detection determined that the frequency of G6PD deficiency among the study population was 9.9%. However, this result does not mean the overall prevalence of G6PD deficiency among the Bangladeshi population because the study was not performed among the general population, rather they were enrolled on the basis of major clinical manifestations including

hyperbilirubinemia or sudden onset of hemoglobinuria or neonatal jaundice accompanied by pallor prior to hospitalization for treatment. Therefore, to determine the overall prevalence of G6PD deficiency among the Bangladeshi population, screening with a large sample size representing people from different territories of the country and different ethnic groups having different phenotypic characteristics as well as genotypic heterogeneity is required.

The present study has identified three non-synonymous point mutations; namely, G6PD Kalyan-Kerala, G6PD Mahidol, and G6PD Orissa. G6PD Orissa (Ala44Gly) mutation is common in tribal population of India and it affects NADP binding at the co-factor binding site of G6PD enzyme [59]. Three-dimensional structural studies of different enzymes have revealed that there are two common variants of NADP binding fingerprint (GXGXXG or GXGXXA) [59, 116, 117]. The peptide stretch spanning from 38 to 44 of G6PD enzyme, namely GASGDLA, corresponds to the GXGXXA dinucleotide (NADP)-binding fingerprint of the enzyme. Alanine to glycine substitution at position 44 creates G6PD Orissa variant which increases the K_m of $NADP^+$ for G6PD Orissa variant because this mutation affects the dinucleotide-binding fingerprint which is essential for coenzyme binding. Alanine residue has a methyl side chain, whereas substituted glycine has a hydrogen atom instead of methyl group. Therefore, the decrease in binding affinity of G6PD enzyme for NADP explains why this mutation causes the generation of moderate level of enzyme activity.

The Enzyme activity level of the patients with G6PD Orissa variant supports the findings of other studies [116, 117]. The reaction rate of $NADP^+$ to NADPH conversion decreases due to this mutation because it increases the thermostability of the G6PD protein. Increased long-term stability of the G6PD protein relies on the formation of tetramer and this process is favored by increased concentration of $NADP^+$[118]. PCR-RFLP method might be helpful to detect the G6PD-Orissa variant because the mutation eliminates HaeIII restriction site (GGCC) in exon-3 [59].

G6PD Mahidol is the most common deficient variant (occurs in 88–96% of G6PD-deficient subjects) in the Thai-Myanmar border area [50, 119, 120]. This variant provides a selective advantage against *Plasmodium vivax* but not against *Plasmodium falciparum*, which stipulates *Plasmodium vivax* has been the driving force for selective advantage conferred by Mahidol variant [121]. G6PD Mahidol variant affects the same codon as does the

Plymouth variant [89]. However, the Plymouth variant with Gly163Asp substitution results in a severe form of enzyme deficiency (<10% enzyme activity) with an acidic amino acid replacing a non-polar amino acid. The G6PD Mahidol variant, (Gly163Ser), where glycine is replaced by uncharged polar amino acid serine, results in a moderate form of enzyme deficiency (10–60% enzyme activity), suggesting that the charged amino acid at position 163 compromises the three dimensional conformation of G6PD protein and the resultant distortion in protein structure affects enzyme activity by reducing protein stability [89].

Although Mahidol and wild type variants do not differ in terms of K_m for NADP+ or glucose-6-phosphate and have similar catalytic efficiency, the thermo-stability of Mahidol variant is less than that of wild type G6PD enzyme and the folding properties of Mahidol protein are also impaired [90]. It has been reported that c.G487A substitution could reduce enzyme activity to 5–32% of wild-type activity which is consistent with this study results [50, 121]. G6PD Mahidol variant creates an Alu I restriction site (AGCT) which can be used to differentiate between wild type and G6PD Mahidol variant using PCR-RFLP method [122].

This study also identified a G6PD Kalyan-Kerala (Glu317Lys) variant, which is one of the three (other two variants are G6PD Mediterranean, G6PD Orissa) most commonly occurring mutations in Indian population [46, 123]. Glu317Lys substitution affects protein structure as the acidic residue is replaced by positively charged basic lysine residue, which is larger in size than glutamate as well. The previous report has demonstrated a decrease in electrophoretic mobility of mutant G6PD protein compared to the wild type protein due to a change in both charge and size of amino acid residue at position 317, indicating disruption of protein conformation [91]. Unlike G6PD Orissa and Mahidol variants, the Kalyan-Kerala variant does not create or eliminate any restriction sites.

G6PD Kalyan-Kerala variant has been reported to show different biochemical properties and this is why the biochemical variants identified in Jamnagar and Rohini were first named as G6PD Jamnagar and G6PD Rohini variants [124]. Subsequently, DNA-based studies identified them as Kalyan-Kerala variant. Hence, it can be concluded that biochemically distinct variants of G6PD could have the same mutation at the DNA level, emphasizing the significance of molecular characterization of G6PD variants. In addition to G6PD Orissa, G6PD Mahidol, and G6PD Kalyan-Kerala variants, other known variants in South East Asia include G6PD Mediterranean (c.C563T), G6PD Viangchan (c.G871A), G6PD Canton

(c.G1376T), G6PD Union (c.C1360T), G6PD Kaiping (c.C1376T), G6PD Gaohe (c.A95G), G6PD Chatham (c.A1003G), and G6PD Vanua Lava (c.T383C) [119, 125, 126]. Importantly, G6PD Mediterranean (c.C563T) is a common G6PD variant in India, Pakistan, and Afghanistan [46, 123, 127, 128]. The absence of this variant in the study population can be attributed to lower genetic variability or a relatively smaller sample size [62]. A study with larger sample size is required to know the actual numbers of G6PD variants in the country.

Chances of drug-induced hemolysis in G6PD deficient individuals differ depending on deficient variants as they impose different types of severity [57]. Thus, it is important to know the G6PD status and G6PD deficient variants prior to antimalarial drug administration because both hemizygous male and homozygous females with severe deficiency as well as heterozygous females with significantly low level of enzyme activity are at risk of drug-induced hemolysis [119]. It has been reported that *Plasmodium vivax* and *Plasmodium ovale* malaria patients who have Mahidol variant of G6PD deficiency can be treated safely by administering a 3-day course of chloroquine followed by a daily dose of 15 mg of primaquine for 14 days [129]. The antimalarial drug dose used in Bangladesh is higher than the WHO recommended dose (0.25 mg/kg) [130].

In Bangladesh, *P. falciparum* infected patients are treated with the combination of artemether-lumefantrine plus single-dose primaquine (0.75mg/kg on day2), whereas patients with *P. vivax* infections receive chloroquine plus 14 days primaquine (3.5mg/kg total over 14 days) for treatment. These treatments are prescribed to patients with malaria infections without considering G6PD deficiency status in the country, which may cause life-threatening conditions. There is thus an impending danger of drug-induced hemolytic anemia, and perhaps even fatality, in deficient patients [131].

The incidences of plasmodium-infected cases in Dhaka city over the years are relatively low because the city falls within the low malaria-endemic area. In the present study, *Plasmodium* infection status of the study participants was not checked. However, malaria is endemic in southern parts of Bangladesh including Bandarban, Rangamati, and Khagrachari and knowing the G6PD deficient status in those malaria-endemic areas would help to treat malaria patients. Therefore, it is essential to extend G6PD deficiency study in malaria-endemic areas of the country.

In addition, the importance of screening for G6PD deficiency in donor blood prior to transfusion to a recipient during infection or treatment with an oxidative drug cannot be neglected, because blood transfusion from G6PD deficient donor may have adverse effects on vulnerable recipients [132].

4.3. Establishment of HRM-based method for rapid and reliable detection of G6PD variants in Bangladeshi females

The third objective of the study was to illustrate the use of the approach, known as high-resolution melting (HRM) curve analysis to supplement G6PD enzyme assay which can increase the detection rate of G6PD heterozygote females targeting the previously reported and unreported G6PD genetic variants in Bangladeshi population. Extensive genetic researches have been revealing huge useful information about genetic abnormalities including chromosomal aberration, overexpression of genes, or mutations of bases. Sensitive, accurate and timely identification of some SNPs or genetic variants can influence clinical decision- making, such as in prescribing personalized medicine that may potentially lead to a better prognosis. In some cases, the detection of SNPs or genetic variants may help to avoid disease-specific conditions, thereby offering an appropriate patients' management strategy.

However, it is important to find a method for the detection of genetic variants which is reliable, time-saving, and cost-effective and uses a high-throughput operational platform to supplement the conventional methods which may be good for diagnosis but they have limitations with higher failure in detection rates in specific conditions. In most cases, to target the gene-encoded products for the diagnosis of a genetic disease, such as fluorescent spot test or measurement of enzyme activity for the detection of G6PD deficiency, as it is less cumbersome, cost-effective and quick.

Unfortunately, targeting the protein product of a gene sometimes can cause an unexpected clinical outcome without offering the real state of the disease. This situation may happen in females with heterozygous G6PD polymorphisms because of random X-chromosome inactivation results in a mosaic RBC population, namely, G6PD-normal and G6PD-deficient RBCs, which suggests that enzyme activity depends on G6PD-normal to G6PD-deficient RBC ratio [133, 134]. Thus, this part of the study aimed to illustrate the importance of high-resolution melting (HRM) curve analysis as a supplemental approach to G6PD enzyme assay method for the detection of heterozygous females with G6PD

deficiency. The approach could target both identified and unidentified mutations responsible for G6PD deficiency among Bangladeshi individuals.

In the present study, the quantitative spectrophotometric G6PD enzyme assay method could detect only 4 out of 63 samples with enzyme activities lower than the cut-off. On the other hand, HRM-based genetic testing that conformed to the Sanger's sequencing data revealed that 9 of the 63 participants were carrying G6PD gene variants that could cause G6PD deficiency, eight in heterozygous states and rest one in homozygous state.

Five of the heterozygote samples had enzyme activities that were higher than the cut-off and such a finding could be attributed to the skewed X-chromosome inactivation which could result in lower number G6PD-deficient RBCs compared to G6PD-normal RBCs [133]. Even though these heterozygote females were normal in terms of G6PD enzyme activities, their enzyme activity status might alter due to a change in the ratio of the mosaic population of normal to deficient RBCs in the later stages of life [135]. Thus, these G6PD heterozygous females with normal enzyme activities might be vulnerable to suffer from oxidative stress upon exposure to oxidizing drugs and intake of certain food that induce oxidative stress. Therefore, oxidizing drugs are suggested to prescribe to the patients after knowing G6PD enzyme activity, at least in the high prevalence area of G6PD deficiency.

Once the test for G6PD deficiency and status of the patient is known, further checking of G6PD enzyme status might not be necessary during future treatment for a parasitic infection, such as for malaria infection. As the G6PD enzyme activity might be subjected to change to a low level due to alteration in a ratio of normal to deficient RBC population, these heterozygous females are at risk of accidental G6PD deficiency-related complications [135]. Moreover, recent studies have demonstrated that antimalarial drugs can cause hemolysis of defective RBC population in heterozygous females [136].

Therefore, hemolytic crisis in heterozygous females can be prevented if G6PD heterozygous status is known. Furthermore, newborns of heterozygous G6PD deficient females should be screened for G6PD deficiency to avoid hemolytic crisis. It is a matter of concern that there are 400 million people worldwide with G6PD deficiency [41]. Although G6PD deficiency is known as a male problem, it should be noted here that these males inherit their mutated G6PD gene from their mothers with homozygous or heterozygous G6PD deficient status.

Thus, information about the G6PD status of mothers may help to avoid accidental threats and neonatal problems which may inflict more severe and damaging effects. There is a chance that a baby boy of a heterozygous G6PD deficient mother who never showed the signs and symptoms of G6PD deficiency may inherit the deficient allele.

In such a case, the baby boy with a G6PD deficient allele may look healthy without any clinical manifestations for the first few days of life. However, he may develop symptoms of jaundice which may eventually lead to bilirubin encephalopathy or kernicterus or even death [36, 137]. Also, a female newborn with heterozygous G6PD deficiency had been reported to manifest similar clinical manifestations. In this case, the female newborn with a normal level of serum bilirubin for the first two days of life was found to have hyperbilirubinemia despite normal G6PD enzyme activity [138]. Such a phenomenon was the result of the destruction of G6PD deficient RBCs, although the normal RBC population survived the oxidative stress.

The possible damage caused due to heterozygous G6PD gene mutation does not end here. Extreme hyperbilirubinemia and death were accounted for a G6PD heterozygous female neonate who was also a heterozygote due to (TA)6/(TA)7 promoter polymorphism for uridine diphosphate glucuronosyltransferase 1A1*28 (UGT1A1*28) [139], which is generally not a risk factor for neonatal jaundice or kernicterus [140-142]. But UGT1A1*28 promoter polymorphism along with G6PD deficiency can lead to extreme neonatal hyperbilirubinemia [143]. To date, at least 160 G6PD gene variants are known and these genetic variants are dispersed throughout the G6PD gene [76]. So, it is not practical to target the full-length G6PD gene by HRM curve analysis for mutation screening.

Fortunately, all of these G6PD variants are not present in all geographical locations of the world and only a small fraction of G6PD variants could be found for a specific geographical location and ethnic population. For example, only 9 variants, namely c.A95G, c.G392 T, c.G487A, c.A493G, c.C592T, c.C1024T, c.C1360T, c.G1376 T, and c.G1388A account for approximately 90% of G6PD deficiency in China [74, 144, 145]. Similarly, in the Thai-Myanmar border area, only G6PD Mahidol (G487A) variant accounts for 88–96% of G6PD-deficient subjects [119, 120]. Three G6PD variants, namely G6PD Orissa, G6PD Mediterranean, and G6PD Kalyan-Kerala are commonly found in Indian population [46, 123]. G6PD Mediterranean (c.C563T) is also common in Pakistan and Afghanistan and accounts for approximately 80% of G6PD deficient cases in Pakistan [127, 128].

Thus, the molecular approach using HRM for the screening of female heterozygous G6PD variants would be appropriate for a set of G6PD mutations, which may be common for a certain ethnic background or geographical location. Hence, HRM approach was planned for the screening of G6PD heterozygous females in Bangladesh because only three G6PD variants in the G6PD gene had been reported as the most common in the country [81]. In addition to three commonly occurring mutations, another G6PD variant, namely G6PD Mediterranean (c.C563T) was identified. Thus, only four sets of G6PD gene-specific HRM PCR primers could help to avoid misdiagnosis of G6PD heterozygous females.

In this study, one set of primers was for the detection of G6PD Orissa (c.C131G) mutation and used a new combination of primer set from the previously published study [74]. Moreover, for detection of mutations, such as c.G392 T, c.C274T, c.A95G, c.G487A C406T, c.A493G, c.T517C, c. C519G, c.C592T, c.A835G, c.G871A, c.C1024T, c.C1004T, c.G1340 T, c.C1360T, c.G1376 T, c.G1381A, and c.G1388A published primers were used [74]. However, the aforementioned study screened specimens of the already known G6PD deficient individuals along with their parents.

In this study, specimens of unknown G6PD enzyme statuses were used and identified some heterozygous samples that had normal G6PD enzyme activities. Moreover, the use of primers from the above-mentioned study has been illustrated to detect mutations like G6PD Kalyan-Kerala (c.G949A) and G6PD Mediterranean (c.C563T), which had not been reported by the authors of the cited article. Thus, this HRM approach could screen G6PD variants in heterozygous females with 100% sensitivity and 100% specificity in Bangladesh. Most importantly, this study has illustrated the importance of the supplemental HRM approach in addition to the quantitative enzyme assay method to maximize the chance of detection of G6PD heterozygous females.

4.4. Conclusions

In conclusion, this is the first report from Bangladesh about the establishment of age-specific cut-off limits of amino acids and acylcarnitines for the diagnosis of IEM using LC-MS/MS-based technology. In addition, this study is the first report where mutations in the G6PD gene responsible for the G6PD deficiency in Bangladesh has been thoroughly characterized. Based on the key findings of the study, a rapid, reliable and cost-effective HRM-based technique has been established to detect these G6PD variants in heterozygous females. This study not only established cut-off values for various metabolites for

Bangladeshi population and new HRM-based cost-effective methodology for the detection of G6PD gene variants which causes G6PD deficiency in Bangladesh but also gained practical experience in using these high-throughput approaches and determine its overall efficiency and reliability in a Bangladeshi laboratory setting. Due to the high frequency of consanguine marriage and for lack of any screening in the public sector, IEM and genetic disorders are relatively high in Bangladesh. Initiatives should be taken to create awareness about this critical matter and initiate a nationwide newborn screening program in Bangladesh to decrease these genetic disorders and the consequences on the health of the population. This study will pave the way to the other researchers or government authorities in installing and establishing the new technology for screening of inborn errors of metabolism through the national newborn screening program in Bangladesh.

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CHAPTER 5

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APPENDIX I

Appendix I

Table A1: Intra-assay precision and accuracy analysis of LC-MS/MS method for amino acids and acylcarnitines using low, medium and high control specimens.

Analytes	Low Control			Medium Control			High Control			Average intra-assay CV (%)	Average RE (%)
	Target (µmol/L)	Intra assay mean (µmol/L)	Intra assay CV (%)	Target (µmol/L)	Intra assay mean (µmol/L)	Intra assay CV (%)	Target (µmol/L)	Intra assay mean (µmol/L)	Intra assay CV (%)		
Ala	149..6	128.54	16.72	465.1	357.41	20.34	767	595.87	13.25	16.77	-19.85
Arg	38.7	32.2	5.46	100.6	102.87	2.43	374.6	467.33	0.95	2.95	3.41
Cit	34	30.78	14.82	77.5	75.669	9.84	282.6	322.15	12.01	12.22	0.73
Xle	160	162.61	7.34	483.4	493.84	7.17	767.2	814.62	4.75	6.42	3.32
Lys	80.8	71	4.63	123.9	121.37	3.89	332.9	353.93	2.66	3.73	-2.62
Met	35.7	38.54	12.37	117	110.95	9.08	506.6	475.56	5.47	8.97	-1.11
Orn	57	40.4	10.1	110	99.68	6.99	351.5	389.29	3.77	6.95	9.33
Phe	55.9	57.74	7.08	121.3	123.17	4.89	425	441	3.97	5.31	2.87
Pro	123.7	124.87	3.14	401.5	357.17	5.13	684.6	656.88	3.26	3.84	-4.72
Ser	168.6	153.2	23.68	533.1	566.67	16.07	892.8	897.62	9.03	16.26	-0.77
Tyr	106.4	115.37	33.8	283.2	280.34	12.7	501.5	440.17	22.94	23.15	-1.6
Val	88.1	88.37	11.69	182.4	173.55	9.22	589.5	557.49	4.44	8.45	-3.33
C0	20.57	20.96	12.82	49.5	51.39	6.8	90.6	94.45	5.24	8.29	3.32
C2	24.97	23.63	2.92	67.5	69.66	4.42	127.7	137.27	4.86	4.07	1.77
C3	3.6	3.65	3.79	11.2	11.62	6.4	21.8	22.43	3.82	4.67	2.72
C4	0.16	0.15	9.91	0.76	0.79	4.18	1.5	1.67	5.21	6.43	2.21
C5	0.25	0.23	15.29	1.49	1.64	8.26	3.06	3.57	5.1	9.55	6.93
C6	0.08	0.08	37.06	0.65	0.67	3.61	1.35	1.46	5.73	15.47	3.93
C8	0.13	0.1	90.58	1.01	1.18	10.47	2.12	2.55	6.15	35.73	5.03
C10	0.06	0.08	25.58	0.46	0.46	5.31	0.96	0.94	5.7	12.20	9.00
C12	0.06	0.07	21.11	0.43	0.42	6.71	0.91	0.83	5.03	10.95	1.81
C14	0.12	0.11	8.43	0.45	0.43	7.25	0.87	0.82	4.44	6.71	-6.46
C16	3.85	3.78	1.93	9.24	9.76	2.63	16.67	17.76	1.49	2.02	3.45
C18	0.97	0.98	3.42	1.93	2.06	2.8	3.32	3.49	4.06	3.43	4.29

Abbreviations: RE, relative error; Ala, alanine; Arg, arginine; Cit, citrulline; Xle, (Leucine and isoleucine); Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanin; Pro, proline; Ser, serine; Tyr, tyrosine; Val, valine; C0, free carnitine; C2, acetylcarnitine; C3, propionylcarnitine; C4 butyryl-/isobutyrylcarnitine; C5, isovaleryl-/2-methylbutyrylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C16, palmitoylcarnitine and C18, stearylcarnitine.

Table A2: Inter-assay precision and accuracy analysis of LC-MS/MS method for amino acids, free carnitine and acylcarnitines using low, medium and high control specimens.

Analytes	Low Control			Medium Control			High Control			Average intra-assay CV (%)	Average RE (%)
	Target (µmol/ L)	Inter assay mean (µmol/ L)	Inter assay CV (%)	Target (µmol/ L)	Inter assay mean (µmol /L)	Inter assay CV (%)	Target (µmol/ L)	Inter assay mean (µmol/ L)	Inter assay CV (%)		
Ala	149.6	129.56	6.85	465.1	361.5	14.4	767	596.23	2.3	7.85	-19.31
Arg	38.7	32.37	4.64	100.6	102.86	0.85	374.6	467.37	0.42	1.97	3.55
Cit	34	30.93	7.31	77.5	76.15	5.74	282.6	322.81	1.78	4.94	1.15
Xle	160	163.23	4.63	483.4	492.23	3.71	767.2	812.81	1.98	3.44	3.26
Lys	80.8	71.12	1.58	123.9	121.1	1.94	332.9	353.81	0.45	1.32	-2.65
Met	35.7	38.51	3.38	117	111.28	2.57	506.6	475.17	3.97	3.31	-1.07
Orn	57	40.67	6.2	110	99.88	2.16	351.5	390.43	2.52	3.63	-9
Phe	55.9	57.53	4.31	121.3	122.54	5.17	425	442.09	2.35	3.94	2.65
Pro	123.7	124.71	1.59	401.5	357.15	4	684.6	657.23	1.53	2.37	-4.74
Ser	168.6	156.31	18.21	533.1	571.77	10.1	892.8	894.98	6.48	11.60	0.07
Tyr	106.4	117.31	15.05	283.2	281.14	7.13	501.5	436.47	8	10.06	-1.15
Val	88.1	88.29	2.36	182.4	173.89	7.49	589.5	556.5	2.71	4.19	-3.56
C0	20.57	21.04	6.36	49.5	51.6	3.93	90.6	94.36	1.06	3.78	3.56
C2	24.97	23.64	1.19	67.5	69.58	1.75	127.7	137.58	1.97	1.64	1.83
C3	3.6	3.65	1.08	11.2	11.62	5.14	21.8	22.42	0.88	2.37	2.67
C4	0.16	0.14	9.17	0.76	0.79	2.17	1.5	1.67	2.05	4.46	1.83
C5	0.25	0.23	8.64	1.49	1.64	1.74	3.06	3.58	2.98	4.45	6.66
C6	0.08	0.08	25.33	0.65	0.67	1	1.35	1.46	0.41	8.91	3.45
C8	0.13	0.1	27.9	1.01	1.17	11.05	2.12	2.56	3.47	14.14	4.82
C10	0.06	0.08	24.51	0.46	0.46	3.7	0.96	0.94	4.21	10.81	8.36
C12	0.06	0.07	12.59	0.43	0.42	1.74	0.91	0.83	4.15	6.16	2.19
C14	0.12	0.11	2.93	0.45	0.43	5.02	0.87	0.82	2.53	3.49	-6.61
C16	3.85	3.78	1.25	9.24	9.74	1.69	16.67	17.7	0.53	1.16	3.38
C18	0.97	0.98	2..53	1.93	2.06	0.51	3.32	3.48	3.24	1.88	4.08

Abbreviations: RE, relative error; Ala, alanine; Arg, arginine; Cit, citrulline; Xle, (Leucine and isoleucine); Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanin; Pro, proline; Ser, serine; Tyr, tyrosine; Val, valine; C0, free carnitine; C2, acetylcarnitine; C3, propionylcarnitine; C4 butyryl-/isobutyrylcarnitine; C5, isovaleryl-/2-methylbutyrylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C16, palmitoylcarnitine and C18, stearylcarnitine.

Table A3: Linearity, Limit of detection (LOD), Limit of quantitation (LOQ) and recovery analysis of LC-MS/MS method for amino acids and acylcarnitines using low, medium and high control specimens.

Analytes	R^2	y-Intercept	Slope	LOD	LOQ	Recovery (%)
Ala	0.9997	14.38	0.76	48.53	147.05	80.68
Arg	0.9995	-22.80	1.31	28.91	87.62	103.54
Cit	0.9996	-12.12	1.18	19.61	59.42	101.15
Leu	0.9991	-13.02	1.07	91.65	277.72	103.26
Lys	1.0000	-18.48	1.12	7.10	21.51	97.34
Met	1.0000	4.07	0.93	10.40	31.50	98.92
Orn	0.9999	-29.09	1.19	13.29	40.27	90.99
Phe	0.9999	-2.40	1.05	12.09	36.64	102.65
Pro	0.9954	-3.31	0.95	179.85	545.01	95.25
Ser	0.9954	-3.31	0.95	179.85	545.01	100.06
Tyr	0.9942	39.47	0.80	147.06	445.65	98.85
Val	1.0000	4.69	0.94	11.02	33.38	96.65
C0	1.0000	-0.38	1.05	1.47	4.45	103.55
C2	0.9998	-4.56	1.11	5.97	18.08	101.83
C3	0.9999	-0.01	1.03	0.66	1.99	102.66
C4	0.9993	-0.06	1.14	0.15	0.44	101.82
C5	0.9995	-0.09	1.19	0.26	0.80	106.65
C6	0.9995	-0.02	1.09	0.12	0.37	103.44
C8	0.9999	-0.07	1.24	0.06	0.20	104.82
C10	1.0000	0.02	0.96	0.01	0.03	108.35
C12	0.9994	0.02	0.89	0.10	0.29	102.18
C14	0.9997	0.00	0.95	0.04	0.12	93.37
C16	0.9999	-0.36	1.08	0.57	1.72	103.37
C18	0.9992	-0.03	1.06	0.35	1.07	104.08

Abbreviations: Ala, alanine; Arg, arginine; Cit, citrulline; Xle, (Leucine and isoleucine); Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanin; Pro, proline; Ser, serine; Tyr, tyrosine; Val, valine; C0, free carnitine; C2, acetylcarnitine; C3, propionylcarnitine; C4 butyryl-/isobutyrylcarnitine; C5, isovaleryl-/2-methylbutyrylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C16, palmitoylcarnitine and C18, stearylcarnitine; R^2, Coefficient of determination; LOD, Limit of detection and LOQ, Limit of quantitation

Table A4: Percentile distribution of amino acids, acylcarnitines and related ratios in different age groups of healthy participants.

	Group A			Group B			Group C		
	Percentiles			Percentiles			Percentiles		
	2.50	50.00	97.50	2.50	50.00	97.50	2.50	50.00	97.50
Amino acids									
Ala	74.36	136.60	282.85	88.18	190.95	339.86	111.76	205.15	405.78
Arg	0.50	4.57	21.39	3.26	19.51	74.98	4.26	29.78	95.36
Cit	4.68	9.78	17.17	11.51	20.83	35.91	13.67	24.27	39.74
Xle	70.02	114.62	200.85	75.68	128.43	201.74	102.02	155.75	220.69
Lys	57.34	166.53	416.22	57.39	201.54	295.92	67.03	237.56	379.11
Met	7.51	17.96	42.38	10.61	22.45	40.65	11.69	22.03	42.17
Orn	21.29	55.87	112.77	34.98	86.88	145.61	51.48	94.93	148.49
Phe	27.17	42.54	85.45	32.34	52.40	88.12	33.54	53.72	82.08
Pro	84.25	145.36	320.14	76.18	136.11	256.02	92.59	158.86	324.41
Ser	186.92	339.78	581.31	111.44	198.15	374.39	123.99	209.80	370.87
Tyr	7.11	82.40	199.96	9.19	44.16	128.12	0.37	47.95	136.63
Val	51.55	101.89	188.83	52.78	117.78	187.45	59.58	138.84	210.34
Amino acid ratio									
Phe/Tyr	0.16	0.54	3.47	0.39	1.20	5.11	0.06	1.13	8.03
Val/Phe	1.13	2.37	5.37	1.12	2.11	3.35	1.24	2.52	4.05
Leu/Ala	0.37	0.80	1.37	0.34	0.66	1.44	0.35	0.74	1.23
Leu/Phe	1.43	2.51	5.62	1.53	2.48	3.37	1.83	2.75	4.14
Met/Phe	0.21	0.43	0.74	0.21	0.43	0.77	0.22	0.41	0.78
Met/Leu	0.05	0.17	0.39	0.09	0.18	0.29	0.08	0.15	0.26
Met/Cit	0.72	1.86	4.77	0.47	1.09	2.16	0.47	0.91	1.83
Met/Tyr	0.06	0.23	1.15	0.18	0.48	2.05	0.03	0.47	2.55
Cit/Phe	0.10	0.23	0.42	0.21	0.39	0.75	0.24	0.46	0.80
Cit/Arg	0.53	2.25	16.26	0.31	1.05	5.68	0.30	0.86	4.89
Tyr/Phe	0.13	1.82	5.90	0.19	0.83	2.50	0.01	0.86	2.56
Arg/Orn	0.01	0.08	0.26	0.05	0.21	1.00	0.05	0.36	1.04
Acylcarnitines									
C0	15.60	32.00	73.73	12.34	39.18	69.76	13.38	43.53	75.67
C2	6.88	20.39	64.68	8.71	16.57	34.90	9.05	15.63	29.31
C3	0.61	1.86	6.88	0.63	1.38	2.69	0.64	1.40	2.70
C4	0.08	0.18	0.63	0.06	0.13	0.33	0.05	0.15	0.38
C5	0.09	0.19	0.55	0.04	0.11	0.26	0.05	0.13	0.29
C5:1	0.00	0.00	0.02	0.00	0.00	0.02	0.00	0.01	0.02
C5DC	0.04	0.11	0.29	0.00	0.06	0.17	0.00	0.07	0.20
C5OH	0.08	0.14	0.25	0.12	0.25	0.45	0.13	0.27	0.53
C6	0.00	0.03	0.09	0.00	0.02	0.09	0.00	0.02	0.12
C8	0.00	0.02	0.14	0.00	0.03	0.14	0.00	0.04	0.30
C10	0.03	0.08	0.18	0.00	0.06	0.23	0.01	0.08	0.58
C10:1	0.02	0.05	0.11	0.00	0.05	0.16	0.00	0.07	0.21

C10:2	0.00	0.01	0.02	0.00	0.02	0.08	0.00	0.02	0.08
C12	0.03	0.08	0.23	0.00	0.05	0.14	0.00	0.04	0.15
C14	0.11	0.21	0.51	0.04	0.08	0.27	0.03	0.07	0.13
C14:1	0.04	0.11	0.34	0.03	0.07	0.16	0.03	0.08	0.26
C14:2	0.01	0.02	0.05	0.00	0.02	0.07	0.00	0.03	0.12
C16	0.88	3.07	7.23	0.61	1.19	2.47	0.55	1.15	2.20
C16OH	0.00	0.01	0.05	0.00	0.01	0.01	0.00	0.00	0.01
C16:1OH	0.01	0.03	0.07	0.00	0.02	0.08	0.00	0.02	0.05
C18	0.32	0.69	1.69	0.25	0.47	0.91	0.22	0.40	0.88
C18:1	0.45	0.94	1.98	0.46	0.82	1.57	0.42	0.79	1.67
C18OH	0.00	0.01	0.05	0.00	0.00	0.01	0.00	0.00	0.01
C18:1OH	0.01	0.02	0.03	0.00	0.01	0.02	0.00	0.01	0.02
Acylcarnitines ratio									
C0/(C16+C18)	3.75	8.45	21.67	7.12	23.57	43.54	7.96	27.07	56.08
C3/C2	0.04	0.09	0.19	0.04	0.08	0.15	0.05	0.08	0.14
C3/C16	0.22	0.64	1.42	0.50	1.12	2.37	0.49	1.23	2.36
C3/Met	0.03	0.10	0.33	0.02	0.06	0.17	0.03	0.06	0.15
C4/C2	0.00	0.01	0.03	0.00	0.01	0.02	0.00	0.01	0.02
C4/C3	0.04	0.11	0.28	0.04	0.09	0.27	0.05	0.10	0.27
C5/C3	0.04	0.10	0.42	0.03	0.08	0.16	0.04	0.09	0.19
C5/C0	0.00	0.01	0.01	0.00	0.00	0.01	0.00	0.00	0.01
C5OH/C8	0.00	2.18	165.14	0.00	4.38	72.55	0.00	4.04	87.13
C5OH/C3	0.02	0.08	0.24	0.07	0.18	0.40	0.08	0.20	0.43
C5OH/C0	0.00	0.00	0.01	0.00	0.01	0.02	0.00	0.01	0.02
C8/C10	0.00	0.27	1.67	0.00	0.53	4.43	0.00	0.48	2.45
C8/C2	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.02
C5DC/C5OH	0.28	0.72	1.66	0.00	0.22	0.80	0.00	0.24	1.06
C14:1/C2	0.00	0.01	0.02	0.00	0.00	0.01	0.00	0.01	0.01
C14:1/C16	0.02	0.04	0.09	0.02	0.06	0.16	0.02	0.07	0.22
C16OH/C16	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.01
C18OH/C18	0.00	0.02	0.04	0.00	0.01	0.03	0.00	0.01	0.04

Abbreviations: Ala, alanine; Arg, arginine; Cit, citrulline; Xle, (Leucine and isoleucine); Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Tyr, tyrosine; Val, valine; C0, free carnitine; C2, acetylcarnitine; C3, propionylcarnitine; C4 butyryl-/isobutyrylcarnitine; C5, isovaleryl-/2-methylbutyrylcarnitine, C5:1, tiglylcarnitine; C5DC, glutarylcarnitine; C5OH, hydroxy isovalerylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C10:1, decenoylcarnitine; C10:2, decadienoylcarnitine; C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C14:1, tetradecenoylcarnitine; C14:2, tetradecadienoylcarnitine; C16, palmitoylcarnitine; C16OH, hydroxy palmitoylcarnitine; C16:1OH, hydroxy hexadecenoylcarnitine; C18, stearylcarnitine; C18:1, oleylcarnitine; C18OH, hydroxy stearylcarnitine; C18:1OH, hydroxy oleylcarnitine.

Table A5: Family history and clinical manifestations suggestive of inborn errors of Metabolism (IEMs) among suspected patients with IEM.

Parameters	No. of Cases (N=273)	Percentage (%)
Family history		
Consanguinity	132	48.4
Abnormal birth event	78	28.6
Unexplained death of sibling	38	13.9
Positive family history	40	14.7
Sign and Symptoms		
Seizure	125	45.8
Developmental delay	120	44.0
Lethargy	116	42.5
Irritation	115	42.1
Poor feeding	107	39.2
Abnormal movement	79	28.9
Vomiting	52	19.0
Tachypnea	23	8.4
Toe Walking	18	6.6

Table A6: GC-MS-based urinary metabolic profiling for confirmatory diagnosis of the screening-positive patients with IEMs.

Name of disorder	Case No.	Metabolites detected	Concentration (µmol/mmol of creatinine)	Biological reference interval (µmol/mmol of creatinine)[a]
PKU	Case 1	4-Hydroxyphenylacetic acid	392	3.2 – 180.2
		4-Hydroxyphenyllactic acid	31.6	0 – 8.7
	Case 2	2-Hydroxyphenylacetic acid	52.3	0 – 20
		Phenyllactic acid	11.9	0 – 4
		4-Hydroxyphenylacetic acid	343	3.2 – 180.2
		4-Hydroxyphenyllactic acid	24.9	0 – 8.7
		Mandelic acid	117	0 - 64
	Case 3	2-Hydroxyphenylacetic acid	94.7	0 – 20
		Phenyllactic acid	38.6	0- 4
		4-Hydroxyphenylacetic acid	278	3.2 – 180.2

		4-Hydroxyphenyllactic acid	32.4	0 – 8.7
		Mandelic acid	95	0 - 64
CIT-II	Case 4	Citrulline	18803	10 - 99
MMA	Case 5	Methylmalonic acid	10.4	0 - 6
IVA	Case 6	Isovalerylglycine	91.4	0 - 10
		3-OH-isovalerate	65.3	3.1 23.1
CUD	Case 7	3-Hydroxybutyric acid	108	0 -11.1
		3-Hydroxyisovaleric Acid	64.7	3.1 – 23.1
		Adipic acid	63.2	0 – 35
		p-Cresol	57.8	0 - 11
MCAD	Case 8	Hexanoyl glycine	0.0	0 – 2.9
		Octanoyl glycine	0.0	0 – 0.2
		Phenylpropionyl glycine	0. 0	0 – 0.1
		Adipic acid	0.1	0 - 35
		Octanoic acid	0.0	0 – 7.7
		Sebacic acid	0.0	0 – 12.3
	Case 9	Hexanoyl glycine,	0.0	0 – 2.9
		Octanoyl glycine,	0.0	0 – 0.2
		Phenylpropionyl glycine,	0.0	0 – 0.1
		Adipic acid	0.1	0 - 35
		Octanoic acid	0.0	0 – 7.7
		Sebacic acid	0.0	0 – 12.3

Abbreviations: PKU, phenylketonuria; CIT-II, citrullinemia type II; MMA, methylmalonic acidemia; IVA, isovaleric acidemia; CUD, carnitine uptake defect; and MCAD, medium-chain acyl-CoA dehydrogenase deficiency. [a]Reference ranges were obtained from Human Metabolome Database (Version 4.0)[1].

References

1. Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vázquez-Fresno R et al. HMDB 4.0: the human metabolome database for 2018. Nucleic acids research. 2017;46(D1):D608-D17.