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Applying a multiplexed primer extension method on dried blood spots increased the detection of carriers at risk of glucose-6-phosphate dehydrogenase deficiency in newborn screening program

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A B S T R A C T

Background: Patients with glucose-6-phosphate dehydrogenase deficiency might develop acute hemolytic anemia, chronic hemolytic anemia, and neonatal hyperbilirubinemia when exposed to high levels of oxidative stress. Severe hemolysis may occur in not only patients but also female carriers under certain conditions. However, 80%–85% of female carriers were undetected in an existing newborn screening program because of their wide-ranging levels of enzyme activity.
Methods: We developed a cost- and time-efficient multiplex SNaPshot assay using dried blood spots.
Results: By detecting 21 common mutations in Taiwan and Southeast Asia, the assay could determine 98.2% of the mutant alleles in our cohort of Taiwanese newborns. The 9 undetermined mutant alleles were consequently detected by Sanger sequencing, of which 5 unpublished variations—c.187G > A (Pingtung), c.585G > C (Tainan), c.586A > T (Changhua), c.743G > A (Chiayi), and c.1330G > A (Tainan-2)—were detected. Furthermore, 13% of mild mutations were missed in male infants whose enzyme levels at 6.1–7.0 U/gHb in the newborn screening program when set the cutoff value at 6.0 U/gHb. We therefore suggest increasing the cutoff value and applying the multiplex SNaPshot assay as the second tier for neonatal screening.
Conclusions: Our approach could significantly increase the detection rate of male patients and female carriers with a reasonable cost and a reasonable number of clinic referrals.

1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD; E.C 1.1.1.49, gene symbol: *G6PD*) is the rate-limiting enzyme of the pentose phosphate pathway. Nicotinamide adenine dinucleotide phosphate (NADPH) produced by G6PD is the key substance protecting against reactive oxygen species, and it reduces damage in most cells; furthermore, G6PD is the only source of NADPH in erythrocytes [1]. Therefore, patients with G6PD deficiency are susceptible to oxidative stress and are at risk of hemolytic anemia. Acute hemolytic anemia, chronic hemolytic anemia, and neonatal hyperbilirubinemia may occur spontaneously or may be triggered by infection, stress, hyperglycemia, or certain foods or medications, including fava beans, naphthalene, aspirin, or antimalarial drugs [2–4].

G6PD deficiency is the most common inherited disease worldwide; it is observed mainly in tropical Africa, tropical and subtropical Asia, the Mediterranean, and the Middle East [4]. Although *G6PD* is located on the X chromosome, low enzyme activities are usually observed in hemizygous men but are also observed in female carriers due to lyonization [3,5]. Severe hemolysis may occur in female carriers under certain conditions such as medical stimulation combined with abnormal hepatic function. Because female carriers may exhibit variable enzyme activity from low, borderline, to normal [1,3], only 15%–20% of female carriers were estimated to have been detected using general newborn screening programs [6]. This low detection rate suggests that most of the female carriers are at risk. Therefore, the determination should rely on a method independent of an enzyme activity assay. For a single-gene disorder with common mutations, such as G6PD deficiency, molecular analysis should be the easiest methods to use.

Several methods for detecting G6PD mutations have been reported,

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Table 1
Sequence of extension primers used for G6PD SNaPshot reactions.

Mutation name	cDNA position	Primer sequence (5′ → 3′)	Size (bp)	Ori[a]	Extension (WT/Mut)	Previously observed in Southeast Asia	Selected ref.
TS1 extension group							
Union	1360	(CTGA) GGAGCCAGATGCACTTCGTG	24	+	C/T	Taiwan, Philippines, Vietnam	[15,23,27,28]
Gaohe	95	$(CTGA)_2$ GCCTTCCATCAGTCGGATACAC	30	+	A/G	Taiwan, South China	[23,27,28]
Mediterranean	563	$(TGAC)_3$ TGTCCTCACGGAACAGGGAG	32	−	G/A	Malaysia	[15]
Taipei	493	$(ACTG)_3$ ACTGCTTCTCCACGATGATGCGGT	36	−	T/C	Taiwan	[23]
Chinese-5	1024	$(GACT)_4$ GACCCACCTCTCATTCTCCACATAGA	42	−	G/A	Taiwan, South China	[23]
Vanua Lava	383	$(ACTG)_5$ ACTCTCAACAGCCACATGAATGCCC	45	+	T/C	Indonesia, Philippines	[15,28]
Fushan	1004	$(GACT)_8$ GAGCGGGTCCACCACCG	49	+	C/A	Taiwan, South China	[23]
Coimbra	592	$(CTGA)_8$ CCCAGGTAGTGGTCGATGC	51	−	G/A	Taiwan, Indonesia, Myanmar	[15,23,28]
Quing Yuan	392	$(GACT)_9$ GGCGGTTGGCCTGTGAC	53	−	C/A	Taiwan, Thailand	[23,27]
Mahidol	487	$(ACTG)_{10}$ TCCGGGCTCCCAGCAGA	57	+	G/A	Taiwan, Thailand, Myanmar, Malaysia	[15,23,28]
Viangchan	871	$(TGAC)_8$ TGCTGCACCTCTGAGATGCATTTCAACA	60	−	C/T	Cambodia, Vietnam, Thailand, Malaysia, Indonesia	[15,23,27,28]
Taiwan-Hakka	1376	$(TGAC)_{11}$ TGCCTCAGCGACGAGCTCC	63	+	G/T	Taiwan, South China, Thailand	[15,23,27,28]
Taipei-Hakka	1388	$(GACT)_{10}$ GATGGTGCAGCAGTGGGGTGAAAATA	66	−	C/T	Taiwan, South China, Thailand	[15,23,27,28]
Miaoli	519	$(TGAC)_{13}$ CATCATCGTGGAGAAGCCCTT	73	+	C/G	Taiwan	[23]
TS2 extension group							
Andalus	1361	GACTGAGCCAGATGCACTTCGTGC	24	+	G/A	Malaysia	[15]
−	94	$(GACT)_2$GATGCCTTCCATCAGTCGGATACA	32	+	C/G	Myanmar	[26]
−	551	$(GACT)_4$CAGAGCTCTGACCGGCTGT	35	+	C/T	South China	[25]
Chatham	1003	$(GACT)_8$GACGCGGGTCCACCACC	49	+	G/A	Taiwan, Indonesia, Philippines	[15,28]
Selma	376	$(ACTG)_8$AGCGCCTCAACAGCCACATG	52	+	A/G	Malaysia	[6]
Keelung	1387	$(CTGA)_{12}$AGCTCCGTGAGGCCTGG	65	+	C/T	Taiwan, South China	[23]
Nankang	517	$(CTGA)_{13}$GCATCATCGTGGAGAAGCCC	72	+	T/C	Taiwan, South China	[23]

[a] Direction of probes with respect to template; +:sense; –: antisense.

including restriction fragment length polymorphism analysis [7], the amplification refractory mutation system [8], denaturing high-performance liquid chromatography [9], single-strand conformation polymorphism [10], high-resolution melting assay [11,12], denaturing gradient gel electrophoresis [13], a gold-nanoparticle-based technique [14], and Sanger sequencing [15,16]. However, these methods have low throughput in addition to being labor intensive, time consuming, or both. Recently, new technologies have been applied to mutation analysis. The Sequenom iPLEX Gold assay can accurately and rapidly analyze multiple *G6PD* mutations [17]. However, the high cost of the instrument prevents its extensive application. SNaPshot assays have also been developed to detect common *G6PD* mutations [18–20] and have been reported to rely on the use of genomic DNA as a template; this necessitated recalling families to confirm diagnoses and increased the psychological stress before the confirmation results.

2. Methods

2.1. Subjects

Blood spots were obtained from the Chinese Foundation of Health, which is responsible for screening one-third of newborns geographically distributed in different regions of Taiwan. After the dried blood spots were received, G6PD activity was measured using G6PD reagent obtained from Trinity Biotech Plc. For G6PD activities below a specified cutoff value (currently set at 6.0 U/gHb), the infants, and their parents if the infants were girls, were recalled to the referral hospital, and the diagnosis was confirmed through a quantitative enzyme activity assay by using fresh whole blood. In Taiwan, the quality of all screening and referral laboratories was maintained by an external quality assurance program for newborn screening of G6PD deficiency [21], ensuring that the data collected from different laboratories were comparable. The distribution of G6PD activities of newborns in Taiwan were firstly analyzed among 65,193 newborns (33,980 male and 31,213 female newborns) screened for G6PD deficiency from July 1, 2008, to June 30, 2009, at the Chinese Foundation of Health. Among the same period of time, a total of 1415 dried blood spots of infants whose mothers were Han Chinese were selected and divided into 3 groups: (1) 500 G6PD-deficient male individuals confirmed through a quantitative enzyme activity assay; (2) 90 individuals with G6PD activity > 10.0 U/gHb (52 male and 38 female individuals) included as unaffected controls; and (3) 825 individuals with G6PD activity of 4.1–10.0 U/gHb in newborn screening (141 male and 684 female individuals). The study protocol was reviewed and approved by the Institutional Review Board of Taipei City Hospital, Taiwan.

2.2. Multiplex SNaPshot assay

This study included 14 of the most common *G6PD* mutations in Taiwan [9,22,23] and the other 7 common *G6PD* mutations observed in neighboring areas, such as south China [20,24,25], Cambodia [26,27], Indonesia [28], Malaysia [15], Myanmar [26], the Philippines [28,29], Thailand [28], and Vietnam [27]. DNA was extracted from the dried blood spots collected on a filter paper as previously reported [30]. A one-tube multiplex polymerase chain reaction (PCR) was performed with pairs of primers amplifying DNA fragments containing common mutations in Taiwan and Southeast Asia (Supplementary Table S1) by using the Complete PCR Reagent Set (Agena Bioscience). PCR primers were then eliminated using ExoSAP-IT reagent (Thermo Fisher Scientific) and used as the template for the subsequent primer extension reactions.

Multiplex primer-extension reactions were performed using a SNaPshot Multiplex Kit (Thermo Fisher Scientific). The 20–24 bases of the 3′ region of the extension primers were designed to anneal to the 5′ region of the sense or antisense strand flanking the mutated nucleotide. An appropriate length of tetranucleotide (dGACT) was added to the 5′ end of the extension primers to generate PCR products with variant lengths upon reaction (Table 1). The reaction mixture contained 3 μL of PCR products, 5 μL of a SNaPshot reaction mixture (containing ddNTP terminators, DNA polymerase, and sequencing buffer), and 1 μL of a SNaPshot extension primer mixture. The reaction condition was as follows: 96 °C for 10 s, 55 °C for 5 s, and 60 °C for 30 s for a total of 25 cycles. The reaction with different fluorescently labeled ddNTP terminators allowed single-base extension at the 3′ end of the extension

primers, which would represent the sequence of the common mutation points. The products were incubated at 37 °C for 60 min with one unit of shrimp alkaline phosphatase, followed by heating at 75 °C for 15 min for enzyme inactivation. The products were subjected to capillary electrophoresis on an ABI 3730xl DNA analyzer (ThermoFisher Scientific) and analyzed using the GeneMapper software package (ver 5.0).

2.3. Sanger sequencing

The coding regions and flanking areas of *G6PD* were analyzed for G6PD-deficient patients whose mutations were not determined by the multiplex SNaPshot assay. The DNA sequencing was performed as previously described [30]. Reference accession number NM_001042351.1 was used as the reference sequence. The description of newly discovered mutations follows the nomenclature recommended by Human Genome Variation Society (http://varnomen.hgvs.org/).

3. Results and discussion

3.1. Distribution of G6PD activity of neonates in Taiwan

In total, 65,193 newborns (33,980 male and 31,213 female newborns) had been screened for G6PD deficiency from July 1, 2008, to June 30, 2009, at the Chinese Foundation of Health, 1 of the 3 newborn screening centers in Taiwan. The mean ± SD and median G6PD activities were 12.0 ± 2.9 and 12.2 U/gHb, respectively, in the male newborns and 12.4 ± 2.5 and 12.4 U/gHb, respectively, in the female newborns (Fig. 1A). No statistical differences in G6PD activities were observed between the male and female newborns. When the cutoff value of enzyme activity was set at 6.0 U/gHb, 1094 male and 233 female newborns were determined as being deficient in G6PD. In the subsequent quantitative analysis of G6PD activities in which fresh whole blood was used, 973 male and 184 female newborns were confirmed as being G6PD deficient, 12 male and 24 female newborns were confirmed as negative, and the remaining newborns were either lost to follow-up, refused for confirmed diagnosis, or had a family history (Fig. 1B and C). Therefore, the incidence of G6PD deficiency was approximately 1/35 in male newborns and 1/170 in female newborns in Taiwan. Moreover, one female infant died of severe respiratory infection (G6PD activity: 6.0 U/gHb) before confirmation. We determined that she carried a heterozygous c.95A > G mutation of *G6PD*. Studies have suggested that carrying *G6PD* mutations might increase the severity of certain infections [31,32]; however, whether this infant's death was correlated with low G6PD activities was undetermined.

In the group with G6PD enzyme activities of < 6.0 U/gHb, the median was 1.8 U/gHb in the male newborns (Fig. 1B) and 5.2 U/gHb in the female newborns (Fig. 1C). The enzyme activities of deficient male newborns were different from those of normal controls. The false-positive rate was predominantly observed in newborns with G6PD activities ranging between 4.1 and 6.0 U/gHb (Fig. 1C). The distribution revealed that enzyme activities were ambiguous between female normal controls and patients and/or carriers.

3.2. Development of multiplex SNaPshot assay

A one-tube multiplex PCR was used to amplify the DNA fragments, including the 21 common mutations in Southwest Asia (Supplementary Table S1). The extension conditions were then set as 14 predominant mutations in the TS1 extension group and 7 mutations in the TS2 extension group (Table 1). Compared to previous reports in Taiwan [22,23], which would revealed approximately 92.6% of the mutant alleles using mutations listed in the TS1 panel, and approximately 93.5% of the mutant alleles by analyzing those mutations listed in TS1 and TS2 panels, our results represented high detection rate might caused by the national wide sampling. The signals for each mutation point were discernible from normal controls (Fig. 2A), and both

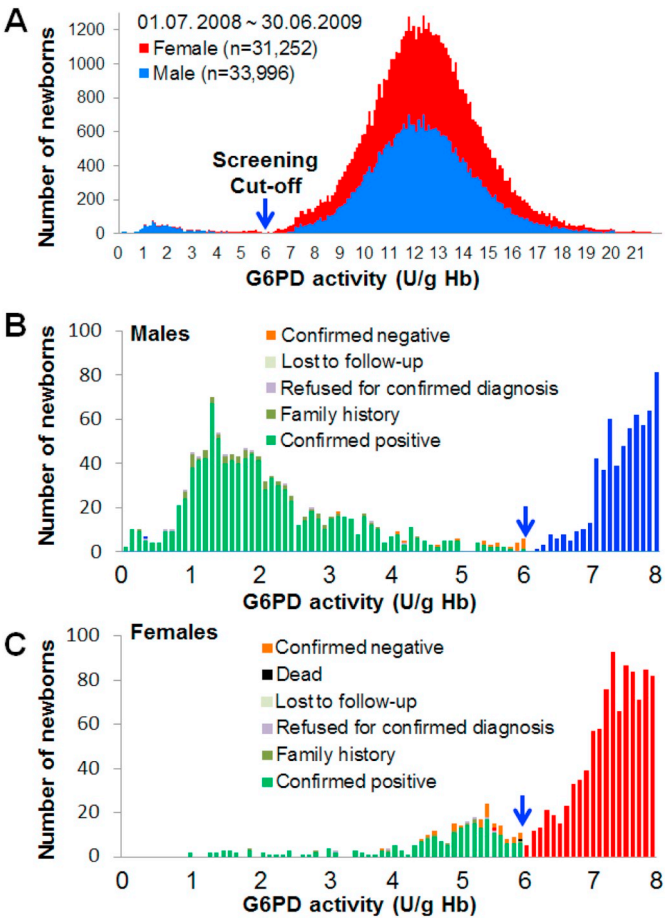

Fig. 1. G6PD enzyme activity distribution in Taiwan. (A) Enzyme activities in male (blue bar) and female (red bar) newborns determined by the Chinese Foundation of Health, one of the 3 newborn screening centers in Taiwan. No significant differences were observed in the distributions between the sexes. The distribution of G6PD activities < 8.0 U/gHb differed significantly between male (B) and female newborns (C). The median was 1.8 U/gHb in male newborns, whereas it was 5.2 U/gHb in female newborns. The false-positive rate increased between 4.1 and 6.0 U/gHb. Blue arrows illustrate 6.0 U/gHb as the current cutoff value. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hemizygous mutations (Fig. 2B and C) and heterozygous mutations (Fig. 2D) could be distinguished.

3.2.1. Detection rate of multiplex SNaPshot in confirmed G6PD-deficient males

The detection rate of multiplex SNaPshot was evaluated in 500 confirmed G6PD-deficient male infants whose mothers were the Han population in Taiwan; 97.2% (486/500) of the mutant alleles could be detected in the TS1 extension group and five mutant alleles (four c.517 T > C and one c.1361G > A) were determined in the TS2 extension group. Nine alleles whose variations were not detected in the TS1 and TS2 panels of our multiplex SNaPshot assay were all detected through Sanger sequencing (Table 2).

After sequencing the coding exons and their flanking sequence of *G6PD* for the nine G6PD-deficient male newborns, we detected eight variations (Table 2). Among these, c.99A > G (p.I33M) [33] was previously not suggested as a disease-causing variation, whereas c.404A > G (p.N63S) [34] and c.697G > C (p.V233 L) [24] were previously reported. The other five variations, namely c.187G > A (p.E63K), c.585G > C (p.Q195H), c.586A > T (p.I196F), c.743G > A (p.G248D), and c.1330G > A (p.V444I), were unpublished. In silico analyses using the SIFT [35], PolyPhen-2 [35], and MutationTaster2

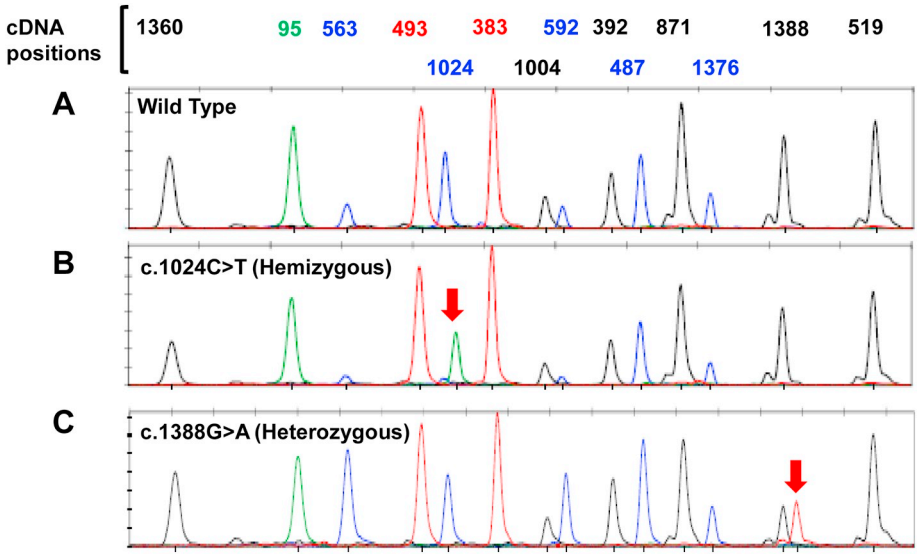

Fig. 2. Multiplex SNaPshot results for (A) a normal control, (B) a male patient with a hemizygous c.1024C > T mutation, and (C) a female carrier with a c.1388G > A mutation. The Fig. represents the discernibility of hemizygous or homozygous (B) and heterozygous (C) mutations. G is denoted in black, A in green, C in blue, and T in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[36], Human Splicing Finder [37] programs as well as the conservation between species and the low allele frequency in the Taiwanese population suggested that all these five were probably disease-causing mutations [38]. This is the first description of these mutations in *G6PD*; we named the five mutations as the Pingtung, Tainan, Changhua, Chiayi, and Tainan-2 mutations, respectively (Table 3).

Previous study suggested that the c.99A > G variation might not affect the enzyme function [33]. One of our patients carrying c.99A > G variations showed G6PD activities slightly below the cutoff values: 4.8 U/gHb in newborn screening and 10.8 U/gHb in quantification confirmation. No other mutation was observed in the coding exons and their boundary regions of *G6PD* in this patient. In addition, the c.99G allele has rarely been observed in the Taiwanese population (2 alleles in 1444 people with indeterminate sex) [39]. Predictions regarding the effects of the mutation were ambiguous: the PolyPhen-2

Table 3
Brief description of five newly discovered G6PD variations.

Mutation name[a]	Nucleotide substitution	Amino acid substitution	Allele frequency[b]
Pingtung	c.187G > A	p.E63K	< 2/1417[c]
Tainan	c.585G > C	p.Q195H	< 1/1000
Changhua	c.586A > T	p.I196F	< 1/1000
Chiayi	c.743G > A	p.G248D	< 1/1000
Tainan-2	c.1330G > A	p.V444I	< 1/1000

[a] Mutation name given in this study.
[b] Allele frequency in Taiwanese population (https://taiwanview.twbiobank.org.tw/browse38 (accessed on 31 August 2018)).
[c] Two alleles in 1417 people with indeterminate sex.

Table 2
Correlation of mutations with G6PD activity in male newborns with confirm diagnoses.

Mutations	G6PD activity (U/gHb) in newborn screening						Frequency	Class[a]
	0–1.0	1.1–2.0	2.1–3.0	3.1–4.0	4.1–5.0	5.1–6.0	(%)	
Detected by multiplex SNaPshot								
c.95A > G	3	19	10	1			**6.6**	**III**
c.392G > T		1	2	6	8	2	**3.8**	**III**
c.487G > A			4				**0.8**	**III**
c.493A > G		13	18	3	1		**7.0**	**II**
c.517 T > C		2	2				**0.8**	**II**
c.519C > G		1	2				**0.6**	**II**
c.592C > T	3						**0.6**	**II**
c.871G > A		3	7	1			**2.2**	**II**
c.1024C > T			2	11	16	3	**6.4**	**III**
c.1360C > T	10				1		**2.2**	**II**
c.1361G > A	1						**0.2**	**II**
c.1376G > T	64	152	15	1	1		**46.6**	**II**
c.1388G > A		25	53	20	2	2	**20.4**	**II**
Detected by Sanger sequencing								
c.99A > G					1		**0.2**	**–**
c.187G > A						1	**0.2**	**–**
c.404A > G					1		**0.2**	**–**
c.585G > C		1					**0.2**	**–**
c.586A > T	1						**0.2**	**–**
c.697G > C		1					**0.2**	**–**
c.743G > A						1	**0.2**	**–**
c.1330G > A						2	**0.4**	**–**

[a] WHO classification of G6PD variants.

program suggested the mutation as benign [35] and the MutationTaster2 program suggested the mutation as disease causing [36]. Further confirmation is necessary to assess the effect of c.99A > G variation for G6PD function.

In summary, a detection rate of 98.2% was observed for both the TS1 and TS2 extension groups (491/500). Similar to previous reports, the c.1376G > T and c.1388G > A mutations were dominant in the Taiwanese population, accounting for 46.6% and 20.4%, respectively. Furthermore, male individuals carrying the c.1360C > T mutation exhibited severe enzyme deficiency, whereas those carrying the c.392G > T or c.1024C > T mutation exhibited mild deficiency (Table 2). Our observations are consistent with those in a previous study [40].

3.2.2. Mutation detection of multiplex SNaPshot in those with high G6PD activities

We evaluated the false-positive rate of our multiplex SNaPshot analysis in 52 male and 38 female infants with enzyme activities of > 10.0 U/gHb. No variation was observed in these 128 alleles when the multiplex SNaPshot assay was used. These data indicate that our multiplex SNaPshot assay can be used with high detection efficiency and a low false-positive rate.

3.3. Detection of mutant alleles in individuals with borderline G6PD activities by using multiplex SNaPshot assay with dried blood spots

3.3.1. Detection efficiency in male individuals with G6PD activities between 4.1 and 8.0 U/gHb

To evaluate the efficiency of the current newborn screening program, we used our multiplex SNaPshot assay to analyze the mutations for male individuals with G6PD enzyme activities between 4.1 and 6.0 U/gHb, including both confirmed positive and negative individuals. Among 81 male newborns analyzed, the detection rate was 90.8% (59/65) in the 4.1–5.0 U/gHb group and 50.0% (8/16) in the 5.1–6.0 U/gHb group (Table 4). No mutation was observed in male newborns confirmed as negative for G6PD deficiency. When we sequenced the samples of all male newborns whose mutations were not found using multiplex SNaPshot analysis, we observed 2 c.406C > T mutant alleles in male newborns confirmed as G6PD deficiency using enzyme assay (Table 4). These data demonstrate the high efficiency of quantitative enzyme assays in confirming male newborns with G6PD activities < 6.0 U/gHb.

When examining 60 alleles in male newborns whose G6PD enzyme activities were between 6.1 and 8.0 U/gHb, we observed 4 mutant alleles in the 6.1–7.0 U/gHb group (Table 4). Although the four mutant alleles were previously classified as mild, namely 2 c.392G > T alleles and 2 c.1024C > T alleles, the observations suggest that some G6PD-deficient male patients would be missed when 6.0 U/gHb is set as the cutoff value.

Table 4
Number of mutant alleles detected in male individuals with 4.1–8.0 U/gHb G6PD activity.

G6PD activity (U/gHb)	G6PD confirmation results	No. of mutant alleles found by multiplex SNaPshot		Subtotal
		NF (%)[a]	Mut. (%)[a]	
4.1–5.0	Positive	2 (3.1)[b]	59 (90.8)	65
	Negative	4 (6.1)	0 (0)	
5.1–6.0	Positive	0 (0)	8 (50.0)	16
	Negative	8 (50.0)	0 (0)	
6.1–7.0	–	26 (86.7)	4 (13.3)[c]	30
7.1–8.0	–	30 (100.0)	0 (0.0)	30

[a] The percentage in the category. NF: not found, Mut.: mutant allele.
[b] The two patients carried c.406C > T, as determined using Sanger sequencing analysis.
[c] The two false-negative patients carried c.392G > T and 2 carried c.1024C > T mutant alleles.

Table 5
Number of mutant alleles detected in female individuals with 4.1–10.0 U/gHb G6PD activity.

G6PD activity (U/gHb)	G6PD confirmation results	No. of mutant alleles found by multiplex SNaPshot			Subtotal
		None (%)	One (%)	Two (%)	
4.1–5.0	Positive	2 (1.9)[a]	96 (92.3)	1 (1.0)[b]	104
	Negative	6 (5.8)	0 (0.0)	0 (0.0)	
5.1–6.0	Positive	1 (1.0)[c]	85 (88.5)	0 (0.0)	96
	Negative	9 (9.5)	1 (1.0)[d]	0 (0.0)	
6.1–7.0	–	31 (16.8)	153 (83.2)	0 (0.0)	184
7.1–8.0	–	54 (54.0)	46 (46.0)	0 (0.0)	100
8.1–9.0	–	63 (63.0)	27 (27.0)	0 (0.0)	100
9.1–10.0	–	89 (89.0)	11 (11.0)	0 (0.0)	100

[a] No mutation was observed through Sanger sequencing.
[b] She carried the c.1024C > T and c.1376G > T mutations.
[c] She carried c.196 T > A mutation, as determined using Sanger sequencing.
[d] She carried a c.1388G > A mutation.

3.3.2. Carrier rate in female newborns with G6PD activities between 4.1 and 10.0 U/gHb

Because of the wide spread of G6PD activities in female carriers, we evaluated the proportion of heterozygotes in female newborns with G6PD enzyme activities between 4.1 and 10.0 U/gHb. Of the 684 female samples analyzed (Table 5), only 1 with 4.6 U/gHb enzyme activity in newborn screening carried 2 *G6PD* mutations, namely the c.1024C > T and c.1376G > T. Most female newborns with G6PD activities between 4.1 and 7.0 U/Hb were actually carriers: 91.3% (95/104) in the 4.1–5.0 U/gHb group, 89.6% (86/96) in the 5.1–6.0 U/gHb group, and 83.2% (153/184) in the 6.1–7.0 U/gHb group. Furthermore, the carrier rate was as high as 46% in the 7.1–8.0 U/gHb group, 27% in the 8.1–9.0 U/gHb group, and 11% in the 9.1–10.0 U/gHb group (Table 5).

When some mild mutant alleles were observed in male newborns with enzyme activities between 6.1 and 7.0 U/gHb (Table 4), female newborns with similar enzyme activities were reasonably considered as at-risk carriers. Approximately 100,000 female babies are born every year in Taiwan, and 0.7% of them show G6PD activities in the range of 6.1–7.0 U/gHb, which indicates that approximately 700 at-risk female carriers are missed every year.

3.4. Comparison of costs of multiplex SNaPshot assay and quantitative enzyme assay

When G6PD deficiencies were observed in male infants in the 6.1–7.0 U/gHb group, adjusting the cutoff value from 6.0 to 7.0 U/gHb would be a reasonable strategy. Considering that 110,000 male and 100,000 female infants are born in a year in Taiwan and that the estimated prevalence of the enzyme activity at 6.1–7.0 U/gHb is 0.2% in male infants and 0.7% in female infants, > 200 male and 700 female newborns would be recalled for quantitative enzyme analysis. The cost of diagnosis confirmation using quantitative enzyme activity assays is US$8 per sample, and the parents of female infants suspected of having a mutation should be included to facilitate the evaluation; recalling all newborns whose G6PD activities are between 6.1 and 7.0 U/gHb would increase the cost by at least US$18,400.

By contrast, our multiplex SNaPshot assay has several advantages. First, the cost of the multiplex SNaPshot assay, including reagents, labor, and instruments, for analyzing both the TS1 and TS2 extension groups is US$7. If the analysis does not require samples of parents of female infants suspected of having a mutation, approximately US$6300

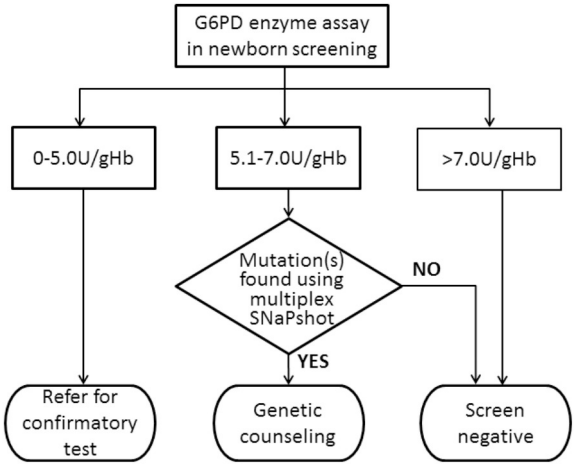

Fig. 3. The recommended flow chart of integrating multiplex SNaPshot assay into current newborn screening program. Based on the results of G6PD activities obtained in newborn screening, the newborns were divided into 3 groups. 1. For G6PD activities below 5.0 U/gHb in newborn screening, the infants and/or their parents were referred for confirmatory test as performed in current procedure. 2. The multiplex SNaPshot assay was suggested applying to all dried blood spots whose G6PD activities are between 5.1 and 7.0 U/gHb. The genetic counseling was recommended to those with mutation(s) found, while the report of screen negative were send to the family when no mutation was found. 3. For those G6PD activities above 7.0 U/gHb, a screening negative result would submit to the family.

would be used for the second-tier method for individuals with G6PD activities between 6.1 and 7.0 U/gHb; this signifies a cost saving approximately US$12,100 compared with the traditional enzyme assay. In addition, the confirmed diagnosis can be directly applied to dried blood spots used in newborn screening without additional clinical referral or parents' whole blood samples; this can thus reduce psychological stress before diagnosis confirmation and the effective cost compared with the regular quantitative enzyme assay. Moreover, the turnaround time for the multiplex SNaPshot assay was estimated to be 24 h, considerably shorter than that of the regular process, including patient referral, blood sample collection, and quantitative enzyme analysis. When the equipment and technique are available, we strongly suggest using the multiplex SNaPshot assay as the second-tier test to facilitate G6PD deficiency screening, especially for those G6PD activities between 6.1 and 7.0 U/gHb.

This study designed primers and probes for multiplex PCR to develop a time-efficient and low-cost multiplex SNaPshot assay method. We could detect mutation hotspots in infants with low G6PD activities and susceptibility to high-oxidant reagents within 8 h. When applied to neonatal screening, the proposed method could increase the detection rate of female carriers, reduce the number of recalls due to temporarily low enzyme activity, and control the number of recalls.

3.5. Challenges of multiplex SNaPshot assay

The limitation of the multiplex SNaPshot assay is that it can detect only selected mutations; therefore, approximately 1.8% of mutant alleles would have been missed in our study populations. When 2 c.406C > T and 2 c.1330G > A mutant alleles were observed in our male patients, including these 2 mutations in the TS2 extension pattern was one of the best solutions to improve our detection rate to approximately 99.0%.

When the multiplex SNaPshot assay was applied to G6PD-deficient male newborns whose mothers were from mainland China or Vietnam, 97.6% (42/43) and 94% (32/34) detection rates could be achieved, respectively. The results demonstrate that the mutation distribution differs between populations, and mutation points should be adjusted on the basis of genetic background.

4. Conclusions

In this study, we developed a cost- and time-efficient multiplex SNaPshot assay using dried blood spots to detect 21 common mutations in populations in Taiwan and Southeast Asia in 2 extension groups. A total of 1415 newborn samples were selected and analyzed. Among 500 confirmed G6PD-deficient male individuals, the mutation detection rate was as high as 98.2% (491/500). No variation was observed in 52 male and 38 female infants with G6PD activities > 10.0 U/gHb. Furthermore, when analyzed 141 male and 684 female newborns with G6PD activity of 4.1–10.0 U/gHb in newborn screening, 13% (4/30) of male patients and 83.2% (153/184) female carries were detected in those enzyme activities between 6.1 and 7.0 U/gHb. These data indicated the risk of miss diagnosis when set the cutoff value at 6.0 U/gHb in current newborn screening program. Considering the accuracy of diagnoses and fragility of newborns with low activities, we recommended increasing the cutoff value to 7.0 U/gHb and directly applying the multiplex SNaPshot assay as the second tier test to all newborns whose G6PD activities are between 5.1 and 7.0 U/gHb in newborn screening (Fig. 3). The multiplex SNaPshot assay can significantly reduce clinic referrals and the psychological stress of families before diagnosis confirmation in patients with G6PD activities between 5.1 and 6.0 U/gHb and increase the detection rate for at-risk newborns whose G6PD activities are between 6.1 and 7.0 U/gHb. Furthermore, the method was cost and time efficient compared to the quantitative enzyme assay.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.04.074.

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