Original Article

Evaluation of Thalassaemia Screening Tests in the Antenatal and Non-Antenatal Populations in Singapore

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Abstract

Introduction: Haemoglobinopathy testing is performed for carrier screening and evaluation of microcytic anaemia. We evaluated the effectiveness of thalassaemia screening tests at our institution and suggest ways of improving the testing algorithm. Materials and Methods: A total of 10,084 non-antenatal and 11,364 antenatal samples with alkaline gel electrophoresis (AGE), capillary electrophoresis (CE), haemoglobin H (HbH) inclusion test, mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) were retrospectively reviewed. A subgroup of 187 samples with genetic testing was correlated with HbH inclusions and MCH/MCV. The effect of iron deficiency on percentage haemoglobin A2 (HbA2) was studied. Results: HbH inclusion test showed low sensitivity of 21.43% for α-thalassaemia mutations but higher sensitivity of 78.95% for $--^{SEA}$ deletion. Byreceiver operating characteristic (ROC) analysis, MCH ≤28 pg or MCV ≤80 fl for non-antenatal samples and MCH ≤27 pg or MCV ≤81 fl for antenatal samples had >98% sensitivity for HbH inclusions. Above these thresholds, the probability that HbH inclusions would be absent was >99% (negative predictive value [NPV] >99%). MCH ≤28 pg had 100% sensitivity (95% CI 95.63%-100%) for α-thalassaemia mutations and 97.68% calculated NPV in the antenatal population. Detection of haemoglobin variants by CE correlated highly with AGE (99.89% sensitivity, 100% specificity). Severe iron deficiency reduced HbA2 in haemoglobin E (*P* <0.001) and α-thalassaemia (*P* = 0.0035), but not in β-thalassaemia. Conclusion: MCH/MCV thresholds have adequate sensitivity for α-thalassaemia in the antenatal population, and genotyping plays an important role as HbH inclusion test shows low sensitivity. CE without AGE, may be used as initial screening for haemoglobin variants. Our study provides contemporary data to guide thalassaemia screening algorithms in Singapore.

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Key words: Haemoglobinopathy, Mean corpuscular haemoglobin, Mean corpuscular volume

Introduction

Haemoglobinopathies are inherited disorders of haemoglobin (Hb) in which the genetic abnormality leads to reduced synthesis of normal globin chains (α- and β-thalassaemia) or functional changes in haemoglobin (haemoglobin structural variants).[1] The haematological parameters of patients with thalassaemia differ widely, ranging from asymptomatic carriers to severe anaemia requiring regular blood transfusion. Globin chain imbalance results in ineffective erythropoiesis, anaemia and microcytosis, the degree of imbalance translating into clinical severity. Deletion of 1 or 2 of 4 alpha-globin genes (–α/αα, – –/αα, –α/–α) causes α-thalassaemia trait; deletion of 3 alpha-globin genes –α/– – or deletions in combination with non-deletional mutation (e.g. $\alpha\alpha^{CS}$/– –) leads to haemoglobin H (HbH) disease; while deletion of all 4 alpha-globin genes (– –/– –) causes Barts hydrops fetalis, a fatal condition in-utero.[2] Alpha^0 denotes 2 gene deletions in *cis* (– –) while alpha$^+$ denotes 1 gene deletion (–α). β-thalassaemia trait arises from inheritance of 1 β-thalassaemia allele, whereas homozygous inheritance of 2 β-thalassaemia alleles leads to thalassaemia major or a moderate form termed 'thalassaemia intermedia'. In Southeast Asia and Singapore, compound heterozygosity for haemoglobin E (HbE) (*HBB*:c.79G>A, β26(B8)Glu>Lys) and β-thalassaemia is a common cause of thalassaemia intermedia.

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Thalassaemia is frequent in the multiethnic population of Singapore which comprises 74% Chinese, 13% Malays, 9% Indians and 3% of other races and where interracial marriages are common. In contrast, sickle haemoglobin (HbS) and sickling disorders are uncommon. In a genotype study of cord blood in Singapore, 6.4% of Chinese, 4.8% of Malays and 5.2% of Indians had α-thalassaemia including Hb Constant Spring (Hb-CS) (overall rate 5.5%). Alpha^{+} deletions accounted for 68% of mutations and $--^{SEA}$ deletion was the commonest type of alpha^{0} deletion.[3] β-thalassaemia including HbE was found in 2.7% of Chinese, 6.3% of Malays and 0.7% of Indians, with β-thalassaemia occurring in 1.6% and HbE in 1.7% overall.[3] Therefore, the major concerns of haemoglobinopathy screening in Singapore are Barts hydrops fetalis and HbH disease in Chinese, HbE/β-Thalassaemia in Malays and β-thalassaemia major in all groups.

Thalassaemia screening is performed either for diagnosis of anaemia and microcytosis or for carrier screening—which is important for assessing a couple's risk of having a severely affected child and at the population level for reducing the burden of thalassaemia major in the community.[4] Prenatal diagnosis and preimplantation genetic diagnosis in ethically allowable settings[5] may potentially be offered to couples at risk.

The International Committee for Standardization in Hematology (ICSH) expert panel in 1978, the World Health Organization in the 1989 Guidelines for the Control of Hemoglobin Disorders, and more recently the Thalassaemia International Federation[6] made recommendations regarding the laboratory investigation of these conditions. Screening is usually performed by a full blood count (FBC) and haemoglobin analysis with quantification of HbA2 and haemoglobin F (HbF). Major haemoglobin variants such as HbS (*HBB*:c.20A>T), haemoglobin C (HbC) (*HBB*:c.19G>A) and HbE are detected as shifted band patterns on Hb electrophoresis. If haemoglobin variant is identified by one technique, a second technique is recommended for positive identification of the variant. However, electrophoresis does not detect all variants, for example, unstable haemoglobins may not be present in sufficient amounts and require detection by heat or isopropanol stability test. Deoxyribonucleic acid (DNA)-based genetic testing is used for unusual or ambiguous results of Hb electrophoresis, cases which require genetic confirmation or can only be confidently detected by genetic testing.

Percentage haemoglobin A2 (HbA2) above 3.5% measured by a robust method such as high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) is the standard for presumptive diagnosis of β-thalassaemia.[6,7] Various factors affect the level of HbA2 in β-thalassaemia, one of which is the type of mutation. For example, mutations which lead to mild reduction of β-globin synthesis may manifest only borderline increased HbA2 of 3.4% to 3.6%, and less common silent β-thalassaemias have HbA2 from 3.1% to 3.5%.[8] Iron deficiency is known to suppress HbA2 levels, and could reduce the sensitivity of the test.[9, 10]

For α-thalassaemia, genetic diagnosis is suggested as the preferred method because of the limited sensitivity of protein-based tests.[1, 6, 11] The commonly used HbH inclusion test, in which red cells are observed for precipitation of unstable β^{4} tetramers, is highly specific for α-thalassaemia and positive in HbH disease, but reported to have poor sensitivity for carrier detection especially in aged samples,[12-14] is time consuming and observer-dependent. Genetic testing is not available in most laboratories and poses cost constraints for large scale screening programmes in high prevalence areas like Singapore. To implement α-thalassaemia genotyping for antenatal screening, screening programmes apply mean corpuscular haemoglobin (MCH) and/or mean corpuscular volume (MCV) thresholds to prioritise patients for screening.[6, 7, 15-18]

Our study aimed to evaluate the effect of applying the above recommendations to thalassaemia screening in our institution. To this aim, we investigated the following: 1) sensitivity and specificity of HbH inclusions (HbH-I) for α-thalassaemia mutations, 2) optimal MCH or MCV thresholds for performing α-thalassaemia testing with HbH inclusion test or α-thalassaemia genotyping in antenatal and non-antenatal populations, 3) determine whether CE alone without alkaline gel electrophoresis (AGE) is sufficient as initial method to detect haemoglobin variants in the Singapore population, and 4) effect of iron deficiency on HbA2 levels as this may impact β-thalassaemia screening.

Materials and Methods

We conducted a retrospective study based on results of adult samples received at the hospital laboratory over a 3-year period from 1 January 2013 to 31 December 2015. The study was approved by the Domain Specific Review Board of the National Healthcare Group. Blood samples from patients submitted to the laboratory for haemoglobinopathy/thalassaemia screening had the following investigations performed concurrently: 1) Haemoglobin gel electrophoresis at alkaline pH (AGE), 2) CE, 3) HbH inclusion test, and 4) FBC with red blood cell (RBC) indices of MCH and MCV. Samples in which 1) and/or 2) detected haemoglobin variant were further subjected to haemoglobin gel electrophoresis at acid pH. Results of serum ferritin were obtained, where available. A proportion of samples additionally had genotyping for α-thalassaemia and β-thalassaemia performed. The decision to perform genotyping was made by the primary physician for reasons such as antenatal cases with unexplained microcytosis, or

haemoglobin variants and positive HbH inclusions which require genetic confirmation. Patients' ethnicity was recorded as the self-reported ethnic group or country of origin. Antenatal samples were from women attending the hospital obstetrics department who were planning for or in their first pregnancy. As per local practice, all women had thalassaemia screening performed, hence antenatal samples approximated unselected population screening.

RBC count, haemoglobin, hematocrit, MCV and MCH were measured on the Sysmex XE5000 automated FBC analyser (Sysmex Corporation, Kobe, Japan). Haemoglobin electrophoresis was performed on agarose gel using the Hydragel System (Hydragel 15 HEMOGLOBIN (E) kit; Sebia Inc., Evry Cedex, France) at alkaline pH 8.6 or acid pH 6.0. The resulting electropherograms were evaluated visually for pattern abnormality by comparing to the reference control. CE of haemoglobin was performed using the automated Capillarys 2 analyser HEMOGLOBIN (E) kit (Sebia Inc., Evry Cedex City, France). This was used to measure the percentages of HbA, HbA2 and HbF as well as any haemoglobin variants, including HbE, HbS, Hb-CS and Hb Barts.

HbH-I stain was made by mixing 1% Brilliant Cresyl Blue (BCB)-staining solution prepared by dissolving 1.0 g of BCB (Sigma-Aldrich, St Louis, Mo) in 100 mL of citrate-saline solution, with K3-ethylenediaminetetraacetic (EDTA) blood in a 1:1 ratio, then incubating in a 37°C water bath for at least an hour. Two smears were prepared, and 10,000 to 50,000 RBCs observed for inclusions. Serum ferritin concentration was measured by chemiluminescent two-site sandwich immunoassay using Beckman Coulter Unicel DXI 800 (Beckman Coulter Inc., Brea, CA, USA).

Genotyping: The alpha-globin gene cluster was examined for 7 deletional mutations ($--^{SEA}$, $-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{FIL}$, $--^{THAI}$, $-[\alpha]^{20.5}$ and $--^{MED}$) by gap-polymerase chain reaction (gap-PCR)[19] and 6-point mutations within the α2-globin (*HBA2*) gene (codon 30 or α30(B11) Glu→0, *HBA2*:c.91_93delGAG); Hb Adana or codon 59 (*HBA2*:c.179G>A [or *HBA1*]; Hb Quong Sze [Hb QS, *HBA2*:c.377T>C]; Hb-CS [Hb CS, *HBA2*:c.427T>C]; Hb Paksé [*HBA2*:c.429A>T] and the polyadenylation [polyA] signal [*HBA2*:c.*92A>G and *HBA2*:c.*94A>C]) by polymerase chain reaction (PCR) and sequencing. PCR amplification of the 3 exons of the beta-globin gene followed by direct sequencing was performed on the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

The following analyses were performed: 1) The sensitivity and specificity of HbH inclusion test for α-thalassaemia was determined by comparing with the gold standard of α-thalassaemia genotyping; 2) To determine the optimal sensitivity and specificity of various MCH and MCV cutoffs for HbH-I, receiver operating characteristic (ROC) analyses were performed in antenatal and non-antenatal samples. In non-antenatal samples, analysis was limited to samples without β-thalassaemia and iron deficiency, i.e. HbA2 <3.5%, HbF <1% and ferritin >30 ng/ml. To preserve better sensitivity, analysis included all samples in the antenatal population; 3) ROC analysis was performed on genotyped samples to determine the sensitivity and specificity of various MCH and MCV cutoffs for genotype-confirmed α-thalassaemia; 4) Results of CE were compared with that of AGE in a 2 x 2 contingency table; and 5) To evaluate the effect of iron deficiency on HbA2, samples were divided into 3 groups by ferritin level: Group 1: ferritin <10 ng/ml (severe iron deficiency),[20] Group 2: ferritin 10 ng/ml to 29 ng/ml (iron deficiency),[21] Group 3: ferritin 30 ng/ml to 200 ng/ml (iron deficiency unlikely). Median HbA2 of Groups 1 and 2 were compared with median HbA2 of Group 3, in the following 4 subgroups: a) samples positive for HbH-I (α-thalassaemia), b) samples negative for HbH-I, c) samples with HbE, and d) samples with HbA2 ≥4.0% (β-thalassaemia). HbA2 ≥4.0% was chosen as these were more likely to have genetic β-thalassaemia, than lower levels.[8]

Data was analysed using GraphPad PRISM Version 7 (GraphPad Software, La Jolla, CA, USA). ROC analysis was used to determine the optimal sensitivity and specificity for continuous variables. Two-by-two contingency tables were used to calculate sensitivity and specificity of categorical data. The Wilson-Brown method was used to compute confidence intervals (CIs) for sensitivity and specificity. Kruskal-Wallis test was used to compare more than 2 groups of non-parametric data. Chi-squared test was used for comparison of proportions. Modified Wald method was used to derive CI of proportions. Statistical significance at a two-tailed *P* value of <0.05 was assumed.

Results

A total of 21,448 samples were analysed, of which 10,084 were non-antenatal and 11,364 were antenatal samples. Genotyping was performed on 187 (0.87%) and ferritin was measured on 5162 samples (24.1%). Figures 1A and 1B show the distribution of all cases and genotyped cases by results. Table 1A shows the demographic distribution and frequency of thalassaemia subtypes, and Table 1B shows the haemoglobin variants detected by electrophoresis among all samples. Figure 2 shows the MCH and MCV of various thalassaemia subtypes.

Correlation of HbH-I with Genotype-Confirmed α-Thalassaemia

Eighty-four cases had at least 1 α-thalassaemia mutation. Table 1C lists the mutations and their correlation with HbH inclusion results. The sensitivity of HbH inclusion test

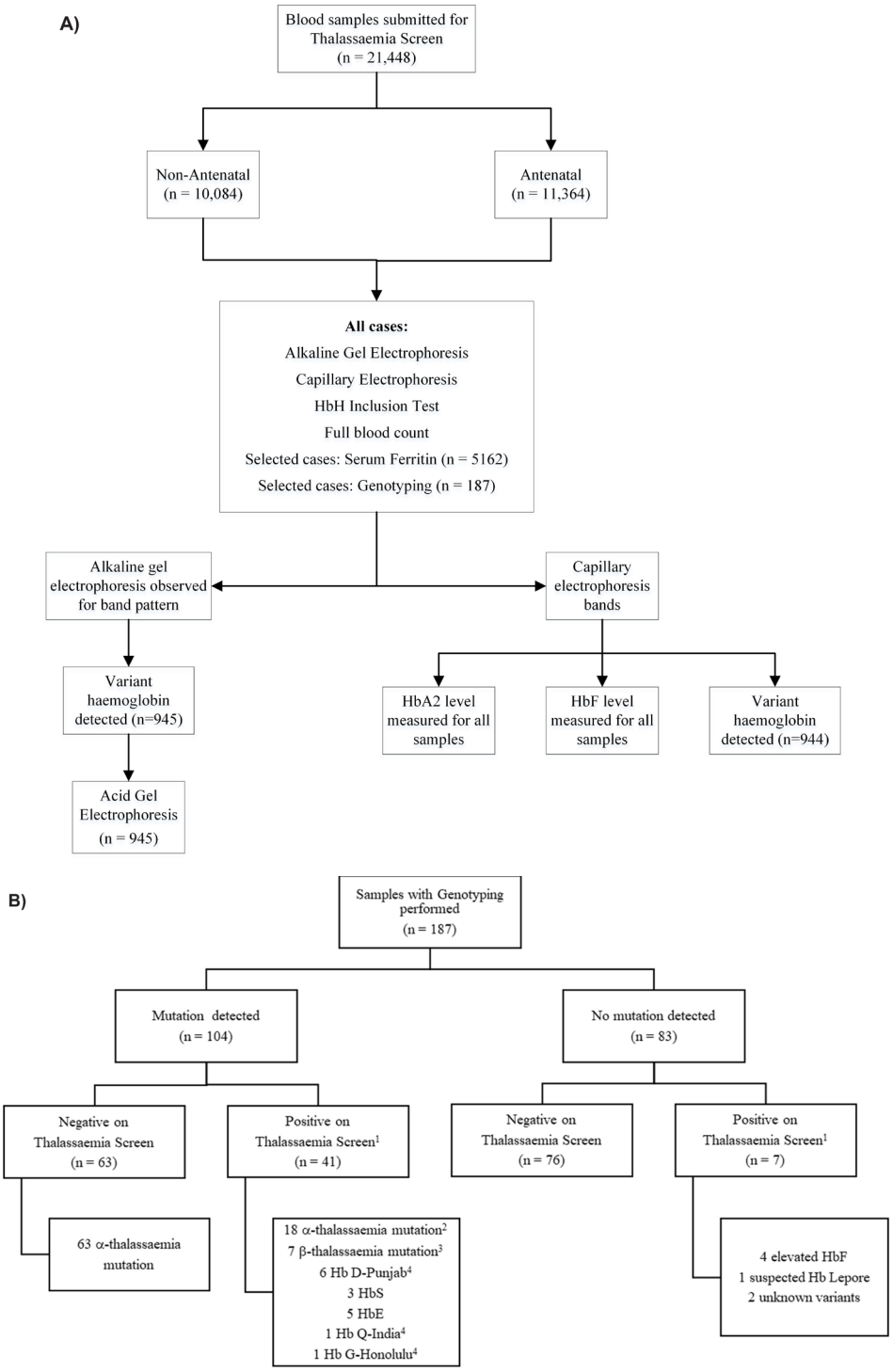

Fig. 1. A) Distribution of samples according to current thalassaemia screening algorithm. B) Distribution of cases by results of genotyping and their correlation with thalassaemia screening tests.[1]Any of the following: HbH inclusion detected, HbA2 >3.5%, variant haemoglobin band on electrophoresis, unexplained HbF elevation. [2]Hb Q-Thailand (*HBA1*:c.223G>C) and Hb Constant Spring (*HBA2*: c.427T>C) are detectable on electrophoresis, hence positive on thalassaemia screen. [3]β-thalassaemia genotypes: IVS1 nt5 (G>C), IVS1 nt2 (T>C), IVS2 nt751 (T>A), codon 41/42 (-TCTT), codon 8/9 (+G) mutation, 619bp deletion, -34TATA (G>A). [4]Hb D-Punjab (*HBB*:c.364G>C), Hb Q-India (*HBA1*:c.193G>C), Hb G-Honolulu (*HBA2*:c.91G>C).

for detecting any α-thalassaemia mutation was 21.43% and specificity was 100.00% (Table 2A). The sensitivity of HbH inclusion test for $--^{SEA}$ deletion was 78.95% and specificity was 98.21% (Table 2B).

Determining Optimal MCH/MCV Cutoffs for HbH-I Testing

By ROC analysis among non-antenatal samples without β-thalassaemia or iron deficiency (n = 2343), MCH and MCV were both highly predictive of HbH-I (area under

Table 1. A) Characteristics of Non-Antenatal and Antenatal Cases, B) Frequency of Haemoglobin Variants Detected by Haemoglobin Gel and Capillary Electrophoresis, C) Alpha Thalassaemia Genotype-Confirmed Cases Correlated with Results of HbH Inclusion Test

A) Characteristics of Non-Antenatal and Antenatal Cases	**Non-Antenatal**	**Antenatal**
Number	10,084	11,364
Age (years), median (interquartile range)	48.6 (34.6 – 63.7)	30.6 (27.5 – 34.0)
Female:male (%)	61.3%:38.7%	100%:0%
Chinese (%)	55.3%	42.2%
Malay (%)	37.1%	16.7%
Indian (%)	6.7%	27.0%
Others (%)	0.9%	14.1%
South-east asian	0.8%	12.4%
East asian	0.0%	0.5%
Middle eastern	0.0%	0.1%
Caucasian	0.1%	1.9%
HbH inclusion positive (%)	15.15%	1.59%
Beta thalassaemia (%) i.e. HbA2 >3.5% without HbE (%)	11.07%	1.21%
HbE (%)	4.76%	1.50%
Others (%)		
B) Frequency of Hb Variants Detected by Hb Gel and Capillary Electrophoresis		
Presumptive Identities of Hb Variants	**No. (Total n = 945)**	**Frequency of Detection**
Hb E trait	715	75.66%
Homozygous Hb E	29	3.07%
Hb Constant Spring	65	6.88%
Hb D-Punjab trait	19	2.01%
Hb S	16	1.69%
Hb Q-Thailand	16	1.69%
Hb Kaohsiung (New York)	13	1.38%
Hb Lepore	9	0.95%
Hb Q-India	4	0.42%
Hb C	0	0%
Hb O-Arab	0	0%
Others	59	6.24%
C) Alpha Thalassaemia Genotype-Confirmed Cases Correlated with Results of HbH Inclusion Test		
Genotype	**HbH Inclusion Detected**	**HbH Inclusion Not Detected**
$-\alpha^{3.7}$ heterozygote	1	41
$-\alpha^{4.2}$ heterozygote	1	2
$-\alpha^{3.7}$ homozygote	0	14
$--^{SEA}$ heterozygote	13	4
$-\alpha^{3.7}/--^{SEA}$ compound heterozygote	2	0
$-\alpha^{3.7}$/Hb Adana compound heterozygote	1	0
Hb Adana heterozygote	0	1
Hb Q-Thailand with $-\alpha^{4.2}$	0	2
PolyA(2) AATAAA>AATA--	0	1
Hb Constant Spring heterozygote	0	1
Total	18	66

Hb: Haemoglobin

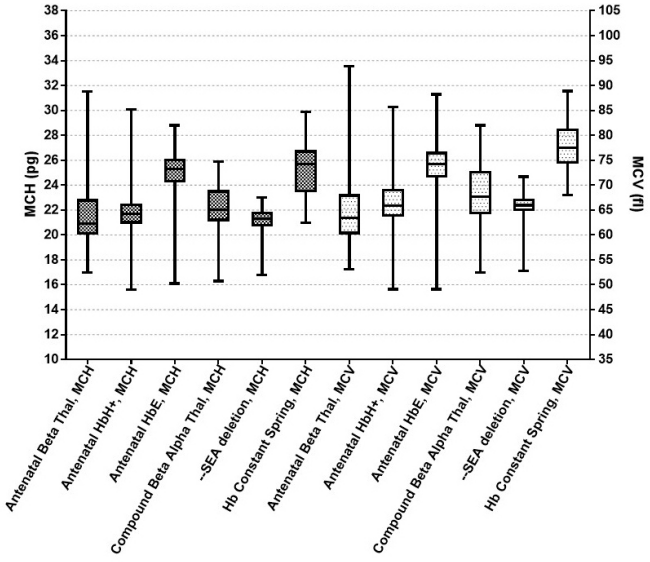

Fig. 2. MCH and MCV of various thalassaemia groups. Data represented as median, interquartile range, minimum and maximum. MCH: Mean corpuscular haemoglobin; MCV: Mean corpuscular volume; Thal: Thalassaemia.

receiver operating characteristic [AUROC] for MCH = 0.9279, P <0.0001; AUROC for MCV = 0.9424, P <0.0001) (Fig. 3). MCH ≤28 pg had a sensitivity of 99.87% (95% CI 99.25%-100%) and specificity of 50.59% (95% CI 48.11%-53.07%) for HbH-I. MCV ≤80 fl had a sensitivity of 99.87% (95% CI 99.25%-100%) and specificity of 63.02% (95% CI 60.61%-65.39%) for HbH-I.

MCH ≤28 pg and MCV ≤80 fl were then evaluated in non-antenatal samples with unknown ferritin and any HbA2 to evaluate their accuracy in unselected cases. The cutoffs showed sensitivities above 98% and negative predictive values (NPVs) above 99% for HbH-I (Table 2C). Positive predictive value (PPV) of MCH cutoff was 19.50% (95% CI 19.11%-19.89%) and PPV of MCV cutoff was 23.96% (95% CI 23.37%-24.57%). Both MCH and MCV had comparable sensitivities (P = 0.2414), but specificity of MCV was higher than that of MCH (P <0.0001).

By ROC analysis among antenatal samples, MCH and MCV were both highly predictive of HbH-I (AUROC for MCH = 0.9398, P <0.0001; AUROC for MCV = 0.9469, P <0.0001) (Fig. 3). Sensitivity, specificity and NPV of MCH <27 pg for HbH-I was 98.90% (95% CI 96.07%-99.87%), 68.65% (95% CI 67.47%-69.81%) and 99.98% (95% CI 99.91%-99.99%). Increasing the MCH cutoff to ≤28 pg did not increase sensitivity but instead reduced specificity to 58.86% (95% CI 57.62%-60.09%). Sensitivity, specificity and NPV of MCV ≤80 fl for HbH-I was 97.24% (95% CI 93.67%-99.10%), 70.28% (95% CI 69.12%-71.42%) and 99.95% (95% CI 99.87%-99.98%). Increasing the MCV cutoff to ≤81 fl increased sensitivity to 98.24% (95% CI 95.23%-99.66%), while NPV remained high at 99.97% (95% CI 99.0%-99.99%). Both MCH and MCV cutoffs had low PPV for HbH-I (5.42%, 95% CI 5.26%-5.60% and 7.26%, 95% CI 6.99%-7.54%, respectively). Hence, MCH ≤27 pg and MCV ≤81 fl were determined to be the best cutoffs for antenatal samples.

Determining Optimal MCH/MCV Cutoffs for Genotype-Confirmed α-Thalassaemia

By ROC analysis among genotyped samples, MCH and MCV were both predictive of genotype-confirmed α-thalassaemia, but with low specificity (AUROC for MCH = 0.6135, P = 0.0069; AUROC for MCV = 0.6104, P = 0.0086) (Fig. 3). MCH ≤28 pg was found to have sensitivity of 100% for genotype-confirmed α-thalassaemia (Table 2D). Using guideline-recommended threshold of MCH <27 pg, the sensitivity for genotype-confirmed α-thalassaemia was lower at 94.05% (95% CI 86.65%-98.04%), 95% CI of the difference: 0.42% to 13.19%, P = 0.024. Using guideline-recommended threshold of MCV <80 fl, the sensitivity for genotype-confirmed α-thalassaemia was 97.62% (Table 2D). The 3 cases with MCH between 27 pg to 28 pg all had MCV ≤81 fl and were single-gene deletions (2 heterozygous $–\alpha^{3.7}$, 1 heterozygous $–\alpha^{4.2}$). The 1 case with MCV >81fl had an MCH of 26 pg and was heterozygous $–\alpha^{3.7}$ plus β-thalassaemia trait.

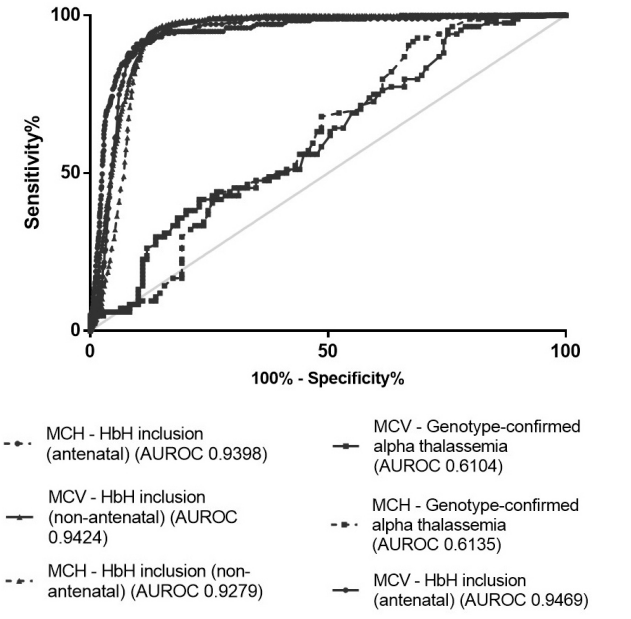

Fig. 3. ROC curves for sensitivity and specificity of MCH and MCV for HbH inclusions in A) antenatal and B) non-antenatal samples. MCH: Mean corpuscular haemoglobin; MCV: Mean corpuscular volume.

Table 2. Two-by-Two Contingency Table for Calculating Sensitivity, Specificity and NPV

Test		Condition Positive (n)	Condition Negative (n)	Test Sensitivity (95% CI)	Test Specificity (95% CI)	NPV (95% CI)
Table 2A		**Alpha Thal Genotype Positive**	**Alpha Thal Genotype Negative**			
HbH inclusion positive		18	0	21.43% (14.01% – 31.35%)	100% (96.40% –100%)	NA*
HbH inclusion negative		66	103	21.43% (14.01% – 31.35%)	100% (96.40% –100%)	NA*
Table 2B		**– –^{SEA} positive**	**– –^{SEA} negative**	78.95% (56.67% – 91.49%)	98.21% (94.88% – 99.51%)	NA*
HbH inclusion positive		15	3	78.95% (56.67% – 91.49%)	98.21% (94.88% – 99.51%)	NA*
Table 2C		**HbH Inclusion Positive**	**HbH Inclusion Negative**			
Sample type	**Test**					
Non-antenatal, unknown ferritin, any HbA2						
	MCH <28 pg	658	2717	99.40% (98.46% – 99.76%)	40.95% (39.53% – 42.38%)	99.79% (99.46% – 99.92%)
	MCH >28 pg	4	1884	99.40% (98.46% – 99.76%)	40.95% (39.53% – 42.38%)	99.79% (99.46% – 99.92%)
	MCV <80 fl	654	2075	98.79% (97.63% – 99.39%)	54.90% (53.46% – 56.33%)	99.68% (99.38% – 99.84%)
	MCV >80 fl	8	2526	98.79% (97.63% – 99.39%)	54.90% (53.46% – 56.33%)	99.68% (99.38% – 99.84%)
Table 2D		**Alpha Thal Genotype Positive**	**Alpha Thal Genotype Negative**			
Sample type	**Test**					
Samples with genotyping performed						
	MCH <28 pg	84	87	100% (95.63% – 100%)†	15.38%* (9.697% – 23.54%)†	NA‡
	MCH >28 pg	0	16	100% (95.63% – 100%)†	15.38%* (9.697% – 23.54%)†	NA‡
	MCV <80 fl	82	87	97.62% (91.73% – 99.58%)†	15.38%* (9.697% – 23.54%)†	NA‡
	MCV >80 fl	2	16	97.62% (91.73% – 99.58%)†	15.38%* (9.697% – 23.54%)†	NA‡
	MCV <81 fl	82	91	97.62% (91.73% – 99.58%)†	11.65%* (6.17% – 19.47%)†	NA‡
	MCV >81 fl	2	12	97.62% (91.73% – 99.58%)†	11.65%* (6.17% – 19.47%)†	NA‡

AGE: Alkaline gel electrophoresis; CI: Confidence interval; Hb: Haemoglobin; MCH: Mean corpuscular haemoglobin; MCV: Mean corpuscular volume; NA: Not applicable; NPV: Negative predictive value; Thal: Thalassaemia

*Specificity may be underestimated, because true negative rates could be higher as samples with MCH >28 pg may not have been genotyped. However, this is not expected to reduce the NPV.

†Lower limit of the 95% CI was used to calculate NPV for antenatal cases.

‡NPV derived from the sample prevalence is not stated as prevalence among genotyped samples is higher than actual population prevalence.

To obtain the NPV and PPV of MCH ≤28 pg and MCV ≤81 fl for genotype-confirmed α-thalassaemia, NPV and PPV in the antenatal population was calculated using the formulae:

$$NPV = \frac{specificity\ x\ (1 - prevalence)}{(1 - sensitivity)\ x\ prevalence + \ specificity\ x\ (1 - prevalence)}$$

$$PPV = \frac{sensitivity\ x\ prevalence}{sensitivity\ x\ prevalence + (1 - specificity\ )\ x\ (1 - prevalence)}$$

and using the lower limit of the 95% CI of sensitivity and specificity, and 5% as the population prevalence of α-thalassaemia. The calculated NPV of MCH ≤28 pg was 97.68% and that of MCV ≤81 fl was 95.70%. The calculated PPV of MCH ≤28 pg was 5.28% and that of MCV ≤81 fl was 4.89%.

Correlation between CE and AGE

There were 945 samples in which haemoglobin variant was detected on AGE. All AGE positive samples, except 1, also had the haemoglobin variant detected on CE. The exception had a faint band which was not further characterised migrating between HbS and HbC zones on AGE but no variant on CE. The sensitivity, specificity, NPV and PPV of CE, using AGE as gold standard, were 99.89%, 100.00%, 99.99% and 100.00%, respectively.

Hb-CS is an important haemoglobin variant to detect on thalassaemia screening. Hb-CS typically appears as a faint slow migrating band on AGE. All 65 cases of Hb-CS which were detected as a slow band on AGE were similarly detected and quantified on CE. The percentages of Hb-CS ranged from 0.2% to 3.1%. Majority of them were HbH-I negative (60 cases, 92.31%). Median (range) of the values were: Hb 12.0g/dl (7.5 g/dl-16.6 g/dl), MCV 77.3 fl (68.0 fl-88.9 fl) and MCH 25.5 pg (19.6 pg-29.9 pg).

Effect of Iron Deficiency on the Percentage of HbA2

HbA2 was significantly lower in samples with severe iron deficiency among HbE positive, HbH-I positive and HbH-I negative subgroups (Fig. 4), with median HbA2 (interquartile range) of each respective subgroup being 3.3% (3.0%-3.6%), 2.2% (2.0%-2.4%), 2.2% (1.9%-2.4%) in samples with severe iron deficiency, compared with 3.6% (3.4%-3.9%), 2.3% (2.2%-2.4%), 2.7% (2.5%-3.5%) in samples without iron deficiency. However, no significant differences in HbA2 was observed in iron deficient samples in the HbA2 ≥4% (β-thalassaemia) subgroup – HbA2 5.1% (4.7%-5.5%) in severe iron deficiency, HbA2 5.2% (4.7%-5.5%) in iron deficiency, HbA2 5.3% (5.0%-5.7%) without iron deficiency.

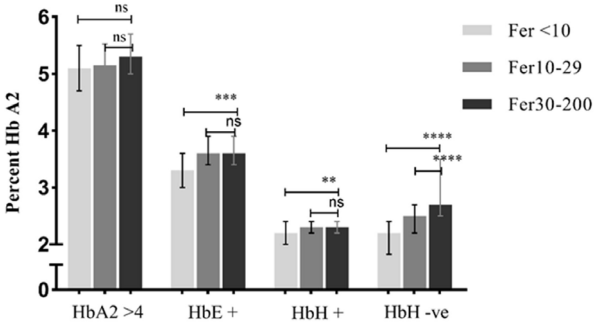

Fig. 4. Effect of iron deficiency on the percentage HbA2 among different thalassaemia subgroups. Data is presented as median with interquartile range; n = 418 in HbA2 ≥4% subgroup, n = 442 in HbE subgroup, n = 238 in HbH+ subgroup, n = 4001 in HbH -ve subgroup. Comparison between groups was performed using Kruskal-Wallis test with Dunn's multiple comparison test. Adjusted *P* value less than 0.0001, 0.001 and 0.001 are indicated as ****, *** and ** respectively. *P* values above 0.05 are denoted as ns: Non-significant. Fer: Ferritin; +: Positive; -ve: Negative.

Discussion

Our study shows that HbH inclusion testing has a low sensitivity of 21.43% for any α-thalassaemia mutation, concordant with other literature.[14, 22] The sensitivity for $--^{SEA}$ deletion is higher at 78.95% but still suboptimal considering that alpha^0 deletion is important to detect for antenatal screening. Even though our results could potentially have underestimated the true sensitivity (as HbH inclusion positive cases may not have been genotyped), it nonetheless reaffirms that genetic testing for α-thalassaemia is essential in cases of unexplained microcytosis. The rate of HbH-I positivity of only 1.59% in our antenatal population compared with 5.5% of α-thalassaemia in the cord blood genotype study[3] corroborates the low sensitivity of the test.

Screening for α-thalassaemia poses challenges because non-genetic tests are insensitive, while genetic tests are costly and unfeasible to implement in all patients. Current antenatal guidelines recommend that patients with unexplained microcytosis directly undergo genetic testing,[6, 15] while other guidelines recommend HbH inclusion test followed by genetic testing and/or partner testing.[17] As screening based on MCH/MCV should have high NPV to reduce misdiagnosis, our study evaluated the predictive values of thresholds. If guideline-recommended thresholds of MCH <27 pg[17] were applied in our antenatal samples, 2 out of 11,364 antenatal samples (0.018%; 95% CI <0.01%-0.08%) would be missed, whereas if MCV <80 fl17 was applied, 5 out of 11,364 (0.044%; 95% CI 0.02%-0.12%) would be missed. However, if MCV <81 fl was used, the number missed would be 3 out of 11,364 (0.026%; 95% CI <0.01%-0.09%). In non-antenatal samples, if MCH >28 pg or MCV >80 fl were taken as a threshold not to screen for HbH-I, 5 out of 10,084 samples (0.049%; 95% CI 0.02%-0.12%) and 10 out of 10,084 samples (0.099%; 95% CI 0.05%-0.19%), respectively would be missed.

In antenatal samples, MCH ≤27 pg and MCV ≤81 fl performed well for predicting HbH inclusions, but MCH ≤28 pg showed excellent NPV for genotype-confirmed α-thalassaemia. It should be emphasised that evaluation should take both MCH and MCV into account because unusual cases of coinheritance of α- and β-thalassaemia may have normal MCV but low MCH, and MCH is a more stable parameter during storage and is less influenced by age.[7, 23]

One study from Thailand found 3 cases of $--^{SEA}$ deletion with MCV >80 fl[24] and a study from Hong Kong illustrated that alpha^{+} deletions can occur with MCV >80 fl.[25] Knowledge of the magnitude of risk of missed diagnosis can inform the decision-making process. In non-antenatal samples, more cases of positive HbH-I occur at high MCH/MCV. We postulate that the reason for this is the higher prevalence of comorbid conditions affecting MCH/MCV, usually by increasing MCH/MCV, such as reticulocytosis, megaloblastic anemia, drugs and liver disease.[16]

Our finding that CE is equivalent to AGE for detecting haemoglobin variants is in agreement with studies comparing CE with AGE which concluded that the 2 methods correlate closely[26] and that CE identified the majority of important haemoglobin variants, i.e. HbS, C, E, D-Punjab, O-Arab, Lepore, without difficulty.[27-29] Both methods are based on separation of haemoglobins by electrophoretic charge and molecular size in alkaline buffer which, although not synonymous, could explain their concordance.

Recent studies found that iron deficiency does not reduce HbA2 to a sufficient degree to affect the diagnosis of β-thalassaemia caused by moderate or severe β-thalassaemia mutations.[10, 30] Our data provides additional reassurance that iron deficiency does not significantly affect the diagnosis of β-thalassaemia in our population. However, the effect on milder β-thalassaemia mutations is not well defined, and since we found that severe iron deficiency significantly reduced HbA2 values in HbE, a mild β-thalassaemic mutation with borderline elevated HbA2 levels,[31] we cannot exclude that other mild β-thalassaemia mutations might be similarly affected. It would therefore be prudent to repeat borderline HbA2 levels (e.g. 3.2%-3.5%) after correction of iron deficiency if time permits.[8] However, in pregnancy when time is of the essence, it may not be possible to repeat testing after treating iron deficiency and proceeding to genetic testing or partner testing may be preferable so that prenatal diagnosis can be offered.[7, 16] The proportion of antenatal patients with borderline HbA2 levels that could potentially be impacted is approximately 2%.

Co-inheritance of α- and β-thalassaemia shifts the MCV and MCH towards normal and lowers HbA2 levels.[32-34] Recent studies showed that in most cases of co-inheritance, HbA2 remains well above 3.5% such that diagnosis of β-thalassaemia is not compromised.[35] Because β-thalassaemia may mask the presence of α-thalassaemia, genotyping for α-thalassaemia is recommended if 1 partner has β-thalassaemia trait and the other is a carrier of alpha^{0} thalassaemia.[33, 36] Finally, other rare causes to consider after thalassaemia and iron deficiency have been ruled out are the δβ-thalassaemias and alpha-globin gene triplication causing microcytosis.[13, 16]

Based on the findings of our study, we propose that the thalassaemia screening algorithm may be simplified. CE and AGE need not be performed together as primary screen, since we showed that both are highly concordant. CE is preferred over AGE as it provides quantification of haemoglobin fractions. Upon detection of a variant on CE, a second alternative, separate method—for example, high performance liquid chromatography (HPLC), isoelectric focusing or electrophoresis in a different medium or pH[16]—should be employed to confirm the identity of the variant. If HPLC is used as primary screen, the same principle applies where a second alternative method should be performed to verify the variant. The choice of methods will depend on the local availability, cost, expertise, ease of use, reproducibility and sample material, whether liquid blood or dried blood spots.[7] If second-line techniques are still unable to confirm the variant or resolve between 2 clinically important variants, then further testing in reference laboratories or DNA analysis is recommended.

In non-antenatal samples, no specific threshold for HbH inclusion testing needs to be applied and all samples may have the test performed, since normocytic samples are more likely to harbour HbH-I (0.05%-0.1% chance) and diagnosis of HbH disease is clinically important in this population. As illustrated in our cohort, 2999 cases (30%) had MCH and MCV above the thresholds, out of which 12 cases harboured HbH-I, including 1 case of HbH disease (Fig. 2). On the other hand, if the clinical impact of α-thalassaemia is deemed low in certain patients, it would be equally possible to omit HbH-I testing in normocytic samples, with the knowledge that non-diagnosis rate is low.

In antenatal samples, both MCH and MCV should be assessed and presence of MCH <27 pg or MCV <81 fL may be used to prioritise samples for HbH inclusion test, since normocytic samples have a lower likelihood of harbouring HbH-I (0.02%). As 7950 samples (69%) in our cohort have MCH and MCV above those thresholds, this strategy (compared to universal HbH testing) would reduce the need for HbH inclusion testing by 60% to 70% with significant conservation of resources. Positive HbH-I should be followed by genetic testing to define the genetic lesion (alpha^{+} versus alpha^{0} deletion) to determine the risk of Barts hydrops fetalis, especially if the partner has α-thalassaemia. A negative test in the presence of confirmed or possible α-thalassaemia in the partner should also prompt genetic

testing. Conversely, partners of women with α-thalassaemia should be offered genetic testing if they have MCH or MCV below the thresholds, and cases of β-thalassaemia trait but unusually "high" MCH/MCV should be genotyped for α-thalassaemia if HbH inclusions are negative.

The question of whether all patients with microcytosis but no HbH-I or β-thalassaemia should undergo genetic testing for alpha^0 deletions remains a matter of debate in a high prevalence population like Singapore, given the significant numbers who require to be tested. In our antenatal cohort, 887 patients (7.8%) fulfill guideline criteria of MCH <25 pg for testing. Our study cannot answer this important question and prospective studies looking at outcomes (number of at risk couples detected, hydrops fetalis prevented) are needed in this regard.

Our study had several limitations. Firstly, only 0.87% of cases had genotyping performed, which limited our study of specific genotypes, for example, $--^{SEA}$. Secondly, genotyped cases were also selectively more likely to have microcytosis without HbH inclusions, which will underestimate the specificity of MCH/MCV as most samples with MCH >28 pg would not have been genotyped, but this effect would not significantly alter the sensitivity. Thirdly, our study focused on the thresholds for HbH-I testing and did not study the thresholds required for single alpha-gene deletion and double alpha-gene deletions. Of note, we considered both alpha$^+$ ($-\alpha^{3.7}$, $-\alpha^{4.2}$) and alpha^0 deletions as positive for α-thalassaemia. Traditionally, the target of genetic screening for α-thalassaemia are alpha^0 deletions rather than alpha$^+$ deletions.[6,8] The Thalassaemia International Foundation recommends that genetic diagnosis be performed if both parents have MCH <25 pg, with the aim of detecting alpha^0 deletions, to reduce anxiety and the burden of genetic testing.[6] Our limited analysis of the 19 samples with $--^{SEA}$ deletions in our cohort revealed that all had MCH <25 pg (Fig. 2). Fourthly, as this was a retrospective study, genetic confirmation was not performed in most of the discordant cases. We cannot exclude false positive HbH due to operator over-reporting, other unstable haemoglobins (e.g. Hb Gun-Hill)[37] or acquired HbH disease occurring in myelodysplastic syndrome.[38] The strength of our study is the large sample size of 21,000 which allowed for narrow CIs. Our sample of approximately 11,000 antenatal samples allowed us to determine the optimal MCH/MCV cutoffs for HbH-I testing with a NPV of more than 99%.

In summary, our study provides contemporary data from a large Singapore cohort to help inform thalassaemia screening algorithms in Singapore.

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