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ORIGINAL ARTICLE

Multiplex ligation-dependent probe amplification (MLPA) assay: a single centre experience of MLPA assay for alpha thalassaemia diagnosis

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Abstract

Introduction: Individuals with alpha(α)-thalassaemia usually have evidence of microcytosis but showed normal haemoglobin A2 and F, except those with three or four gene deletions or those with abnormal Haemoglobin (Hb) such as Hb Constant Spring (HbCS). Definitive diagnosis requires molecular analysis. Multiplex amplification refractory mutation system (ARMS) and gap PCR are reliable for detecting common α-gene mutations; however, many rare or novel mutations remain unidentified. Using principle of primer-specific amplification, abnormality analysed is primer-dependent. This study aimed to compare the detection of HBA gene rearrangements by multiplex ligation-dependent probe amplification (MLPA) with multiplex PCR (ARMS and Gap). *Materials and Methods:* MLPA facilitates amplification of multiple nucleic acid sequences with a single primer pair via identical end probe amplification, thus giving wide α-globin analysis in a single experiment to provide high-resolution detection. Amplification products only require capillary electrophoresis separation followed by software analysis. Seventy-three samples that have been analysed by multiplex PCR were selected for this study. Fifty-five confirmed cases of α-thalassaemia and 18 normal samples were tested using MLPA. Discordant cases suspected of α-thalassaemia underwent sequencing analysis. *Results:* All normal samples and 50 positive cases showed consistent findings between both methods. MLPA showed 100% sensitivity and specificity in detecting HbCS mutation. However, MLPA could not determine zygosity of three homozygous HbCS cases detected by multiplex PCR. The concordant rate was 93.2% between both methods. MLPA results in five discordant cases. *Conclusion:* MLPA is a reliable and accurate technique for characterising HBA gene rearrangements. Overall, both methods showed excellent concordance rate and statistically good agreement. The simplicity of wide α-globin cluster analysis makes MLPA as favourable diagnostic method for the detection both common and unresolved HBA gene abnormalities involving HBA gene cluster.

Keywords: α-thalassaemia, molecular analysis, multiplex PCR, MLPA

INTRODUCTION

Alpha (α)-thalassaemia is the most common inherited disorder of haemoglobin (Hb) synthesis worldwide, affecting up to 5% of the world's population.[1] It is caused by deletions or mutations within the α-globin gene complex, leading to a decrease or absence of α-globin subunits. As in many Southeast Asian countries, *α*-thalassaemia is a public health concern in Malaysia, with prevalence ranging between 10-15%.[2-5] Normal individuals have four α-globin genes (αα/αα). Individuals with one or two α-gene deletions are usually asymptomatic.[6] Problem arises when it interacts with other thalassemia genes or diseases

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since it will alter the disease phenotype and become clinically important. There is a 25% risk of a pregnancy with Hb Bart's hydrops fetalis in couples who are both α° thalassemia carriers (αα/_ _). This is a fatal condition and is accompanied by serious maternal complications. Therefore, screening and identification of the affected person is crucial, since antenatal diagnosis and genetic counselling can be offered to affected families.

Alpha-thalassaemia carriers cannot be detected by conventional screening methods using gel electrophoresis, high-performance liquid chromatography (HPLC) or capillary zone electrophoresis (CE). Definitive diagnosis requires molecular DNA analysis. Setting up of preventive programs requires the establishment of rapid and easy molecular techniques for definitive diagnosis of α-thalassaemia, an important prerequisite for genetic counselling to prevent its deleterious complications. Various methods have been used which include multiplex gap-PCR,[7] Southern blot analysis (SB),[8] multiplex amplification refractory mutation system (ARMS) assay[9] and sequencing analysis.[10] Multiplex PCR (gap and ARMS) allows rapid detection of deletion and non-deletion α-gene abnormalities. However, the applicability requires the definition of the breakpoints, thus limited to known and well-defined deletions. Multiplex PCR (gap and ARMS) is currently used in our centre for α-thalassaemia screening. In case of deletion types, it was performed based on the method described by Chong *et al*.[11] However, based on molecular characteristic among our population, we limited our initial screening panel into three commonest deletions including _α3.7, _α4.2 and _ _SEA compared to seven α-thalassaemia deletion described by Chong *et al*.[11] Only those with negative analysis in the initial screening panel will be further tested for additional 4 deletions consist of _α20.5, _ _MED, _ _Thai, and _ _Fil. For point mutation, it consists of screening for Hb CS, codon 125/ Hb Quang Zhe, codon 59/Hb Adana, initiation codon, codon 30 and codon 35 following the method introduced by Eng *et al*.[9] Of all analyses performed in our centre since October 2010, only 46% cases were confirmed positive using the method, while the remaining 54% cases remain undetected.[12] More than 90% of these groups demonstrated thalassaemic indices. This method has been shown to miss several uncommon cases. These uncommon types are expected, resulting from global multi ethnic migration in Malaysia. As an example, we have found a rare case of α2 gene mutation which was not detected using our panel of PCR primers. Diagnosis was made after being referred to a foreign reference laboratory which has a cost implication on the patient.[12]

A simple technique suitable for rapid quantitative analysis, multiplex ligation-dependent probe amplification (MLPA), was described for the first time in 2002.[13] It is a method which makes a nucleic acid sample suitable for a multiplex PCR reaction, in which up to 50 specific nucleic acid sequences are amplified simultaneously using a single PCR primer pair. This method has been applied successfully in a number of genes in which deletions and duplications are common mutation types.[13,14] It shows a high detection rate ranging from 80-99%.[14] Kipp *et al*.[15] demonstrated 99% concordance between interpretable MLPA and Southern blot results suggesting that MLPA is an accurate technique for diagnosing α-thalassemia. As MLPA has been adapted for high-resolution mapping of deletions, it allows identification of both common and unknown α-globin gene rearrangements to become more effective.[16-18] Furthermore, the simplicity and high accuracy of MLPA in assessing total gene dosage make it a favourable method for thorough characterisation of α-thalassemia mutations. The aim of this study was to use MLPA technique for the detection of α-gene abnormalities. In this study, we determined normal α-gene dosage using MLPA technique on normal control. We also optimised the MLPA methods for the detection of common α-thalassemia gene mutations and we compared the performance of MLPA versus multiplex PCR (gap and ARMS).

MATERIAL AND METHODS

Patient and sample selection

The study was performed using archival DNA material extracted from peripheral blood anti-coagulated with EDTA previously carried out in the Molecular Genetic Laboratory Unit, University Kebangsaan Malaysia Medical Centre (UKMMC) between January 2012 to May 2016 for the diagnosis of α-thalassaemia. Sample size calculation was using Fleiss' kappa for a 95% degree of confidence. Fifty-five patients confirmed positive for α-gene deletion and mutation by multiplex PCR (gap and ARMS) method were selected. Eighteen normal individuals were also selected to be used as normal controls during each MLPA run.

They were identified based on their normal red cells parameters without evidence of deletional or non-deletional α-gene abnormalities which was also performed by multiplex PCR method. The information regarding molecular analysis as well as the age, gender, red cells parameters and Hb analysis of the patients were retrieved from Integrated Laboratory Management System (ILMS). Retrospective analysis was performed for complete blood counts result specifically identifying the Hb concentrations, red blood cells count (RBC) and red cell indices (MCV and MCH). These results were previously determined using an automated blood cell counter Coulter DxH 800 (Beckman Coulter, Miami, USA) and some were tested using Sysmex Xn 3000 (Sysmex, Norderstedt, Germany). The quantitation of HbA2, HbA and HbF obtained using automated Capillary Zone Electrophoresis (CE, Sebia, Inc, Norcross,GA) were also identified to correlate with multiplex PCR analysis. Types of deletion and mutation of confirmed α-thalassaemia patients were selected randomly based on the commonest abnormalities in our centre. These include heterozygous _α3.7, _α4.2, _ _SEA and Constant Spring(ααCS), homozygous _α3.7, compound heterozygous _α3.7/_ _SEA, _α4.2/_ _SEA, _ _SEA/ααCS and Hydrops Fetalis _ _SEA/_ _SEA. For non-deletional type, only ααCS types were included as MLPA was only designed for ααCS mutation.[19] Quality and concentration of selected archived genomic DNA were all assessed by Nanodrop Spectrophotometer GE NanoVue Plus (GE Healthcare, UK) at 260 and 280 μm wavelengths. The samples with low quality were excluded from the study and only those within ratio of 1.8-2.0 were selected to ensure the nucleic acid purity.

MLPA assay

The MLPA reaction was performed according to the manufacturer's instructions.[20] It was carried out using the SALSA MLPA KIT HBA140-C1 (MRC-Holland, The Netherlands). This SALSA MLPA probemix P140 HBA kit contains 45 MLPA probes with amplification products between 130 and 481nt, consist of 34 probes for the HBA gene region and 11 reference probes that detect sequences outside this region. One of these 45 probes (136nt) is specific for the presence of the ααCS mutation. MLPA reaction was executed in four major steps based on the MPLA protocol which includes denaturation, hybridisation, ligation followed by PCR amplification (MRC Holland). The reaction was performed using 5μL (50ng) of DNA samples. In each MLPA experiment, three reference samples of normal DNA were included for comparative purposes and one run without DNA was used as a negative control. Separation of amplification products was subsequently performed using capillary electrophoresis ABI PRISM 3730 (Applied Biosystems, USA). The obtained data were then analysed using Coffalyser Analysis Software, which was an MLPA analysis tool. Relative size of the fluorescent peaks within each sample reflects the relative copy number of the probe's target sequence in the analysed sample. These were compared to reference samples. The MLPA peak pattern of a DNA sample without genomic abnormalities should be identical to that of reference samples. A ratio of ~1 should be obtained if all alleles are present. A deletion of one or more exons in a patient thus becomes apparent as a decrease in relative peak area of the amplification products of the probes corresponding with these exons. For heterozygous deletions, probe ratios will be ~0.5. This final probe ratio was also called Dosage Quotient (DQ). Coffalyser.Net calculates the DQ for each probe in each sample. Threshold ratio for deletion and duplication were set at <0.7 and >1.3 respectively. Discordant cases suspected to have α-thalassaemia but demonstrated inconclusive mutational screening by conventional multiplex PCR and MLPA analysis were further subjected to Sanger sequencing method detecting both HBA1 and HBA2 α-globin cluster gene spanning from -50 of the 5' untranslated region to Poly A of the 3' untranslated region.

Statistics

A statistical analysis was performed using SPSS version 23 (IBM, Armonk, USA). The data were analysed to look for the concordance rate between the two methods, MLPA and multiplex PCR. The kappa value was calculated to evaluate the degree of agreement between the two methods. Kappa value of 0.6 to 0.8 was denoted as good agreement while value of 0.8 to 1.0 was taken as very good agreement. Value below 0.2 indicates poor agreement.

RESULTS

A total of 73 patients, 55 (75%) patients with α-thalassaemia and 18 (25%) normal individuals were analysed using MLPA technique. A total of 7 batches were performed, each batch comprises 12 samples which include 8 known α-thalassaemia cases, 3 normal controls as references and

1 negative control (with no DNA included). Types of deletion and mutation comprise of 12(16%) cases of heterozygous αα/_α3.7, 8(11%) heterozygous αα/_α4.2, 10(14%) heterozygous αα/_ _SEA, 5(7%) cases of heterozygous αα/ααCS, 4(5%) homozygous _α3.7/_α3.7, 5(7%) cases of Hydrops Fetalis with _ _SEA/_ _SEA, 5(7%) compound heterozygous _α3.7/_ _SEA, 1(1%) compound heterozygous _α4.2/_ _SEA, 2(3%) compound heterozygous _ _SEA/ααCS and 3(4%) homozygous ααCS/ααCS.

The protocol was initially optimised using 6 wild-type samples and 8 patients with known deletions. MLPA analysis was then performed and interpretable results were obtained in all 73 tested samples. Overall, MLPA and multiplex PCR methods showed a 93.2% of data concordance (Table 1) with kappa value of 0.86 indicating very good agreement. MLPA results in two discordant cases involving a deletional abnormality. Discussion and consultation with MLPA expertise from MRC Holland were done to verify the MLPA results and to exclude false positive abnormalities. Demographic, haematological and molecular data of the patients were summarised in Table 2.

The first discordant case was initially diagnosed to have heterozygous alpha 3.7 deletion (αα/_α3.7) in a patient whose haematological parameters could not be explained by conventional analysis. MLPA results showed two gene deletions (_α3.7/_α3.7) which was more consistent with the patient's phenotype. Furthermore, there was evidence of HBA gene conversion observed in the MLPA analysis. Subsequent sequencing analysis of both alpha globin genes confirmed the diagnosis of homozygous (_α3.7/_α3.7) and HBA1 gene conversion to HBA2.

The second discordant case, whose clinical phenotype was HbH disease but initially diagnosed to just carry two gene deletions (_α3.7/_α3.7) by multiplex PCR. MLPA resulted in compound heterozygous state of _α3.7 with uncharacterised two gene deletion that has similar pattern to Southeast Asian (_ _SEA) type spanning from a region between HBAP1 and HBAP2 gene until exon 3 of HBQ1 gene. We cannot comment on the detail of the α^0 deleted region since we have lost the patient to our follow up therefore further analysis cannot be performed due to limited sample availability. Sample from the patient's family was also not available, hence family analysis was also not possible. Although the exact breakpoint position and deletion lengths could not be determined at the time of the study, MLPA results showed three gene deletions that could not have been picked up by our multiplex PCR in the first place. MLPA showed 100% sensitivity and specificity

Table 1: Comparison of MLPA and multiplex PCR analysis

Multiplex PCR	Total (N)	Concordant	Discordant
Normal (αα/αα)	18	15	0
Silent carrier (3 α-globin genes present)			
αα/α3.7	12	11	1*
αα/_α4.2	8	8	0
Trait (2 α-globin genes present)			
αα/_ _SEA	10	10	0
_ _SEA/ααCS	2	2	0
_α3.7/_α3.7	4	3	1ǂ
Haemoglobin H (1 α-globin genes present)			
_α3.7/_ _SEA	5	5	0
_α4.2/_ _SEA	1	1	0
Hydrops Fetalis (4 α-globin genes deletion)			
_ _SEA/_ _SEA	5	5	0
α gene mutation			
αα/ααCS	5	5	0
ααCS/ααCS	3	0	3#
Total	**73**	**68(93.2%)**	**5(6.8%)**

*MLPA interpret as _α3.7/_α3.7

ǂ MLPA interpret as α3.7/_ (Compound heterozygous_α3.7 with uncharacterised two gene deletion that has similar pattern to _ _SEA type spanning from a region between HBAP1 and HBAP2 gene until exon 3 of HBQ1 gene)

MLPA identify as positive for αα/ααCS mutation

Table 2: Details of haematology parameters and Hb analysis results for all five discordant cases

Type of α gene defect by multiplex PCR	MLPA findings	Hb (g/dL)	MCV (fl)	MCH (pg)	RBC ($\times 10^9$/L)	HbA (%)	HbA2 (%)	HbF (%)	Hb variant
_α3.7/_α3.7	_α3.7/_ _ (Compound heterozygous _α3.7 with uncharacterised two gene deletion that has similar pattern to _ _SEA type spanning from a region between HBAP1 and HBAP2 gene until exon 3 of HBQ1 gene)	7.8	55.6	15.7	4.96	97.7	2.3	0.0	Nil
αα/_α3.7	_α3.7/_α3.7 with evidence of gene conversion	10.0	74.6	23.4	4.3	97.4	2.6	0.0	Nil
aaCS/aaCS	ααCS	9.1	75.9	24.3	3.7	97.3	2.2	0.0	0.5% Hb variant (HbCS) at zone 2
aaCS/aaCS	ααCS	9.7	77.4	22.6	4.3	95.9	2.2	1.9	4.7% Hb variant (HbCS) at zone 2
aaCS/aaCS	ααCS	8.5	68.3	20.2	4.2	95.8	1.8	2.4	4.1% Hb variant (HbCS) at zone 2, 0.3% peak at zone 1

in detecting HbCS mutation. However, MLPA could not determine zygosity of 3 homozygous HbCS cases detected by multiplex PCR, hence the cases were regarded as discordant in this analysis.

DISCUSSION

Alpha thalassaemia is a public health concern in Malaysia. The definitive diagnosis requires molecular DNA analysis. The Malaysian population consists of multi ethnic groups. This diversity contributes to various interactions of α-genotypes causing heterogeneous molecular pathology of α-thalassaemia in Malaysia.[2,4,5,12,21] In the years to come, further interactions may result in novel gene deletions or mutations with a wide spectrum of phenotypes, thus expecting further challenges in making the diagnosis. Therefore, there is a need to develop an easy, sensitive and cost-effective technique that allows comprehensive diagnosis of the disease.

In this study, we described the detection of α-globin gene rearrangement by MLPA and compared the findings with the multiplex PCR method. As summarised in Table 1, interpretable MLPA results were obtained in all 73 samples subjected to the analysis. MLPA and multiplex PCR method showed a 93.2% concordance

rate, suggesting that the method is an accurate technique for diagnosing α-thalassaemia. This finding was comparable with previous study reported by Kipp *et al*[15] in which they found 99% concordance between MLPA and SB. Although it compared MLPA with a different diagnostic tool, both support the applicability and reliability of MLPA as a method for α-thalassaemia diagnosis.

Ever since first described for α-thalassaemia diagnosis in 2005, development of MLPA as a diagnostic tool to assess deletion and mutation involved α-globin gene has been widely published. It effectively identified both common and unknown α-globin gene rearrangements that remain undetected or unclear using conventional methods such as multiplex PCR, specific PCR and DNA sequencing.[16,17,18,23] Amplification of the α-globin gene cluster for diagnosis is challenging due to considerable sequence homologue within the α-globin gene cluster. In MLPA, ligation dependent probe amplification PCR is carried out using the kit that contains multiple MLPA probes, analysed in one single reaction. By using multiple probes as described, MLPA thus allow multiples nucleic acid sequences to be amplified simultaneously, provide high-resolution identification of deletions/duplications in the α-globin gene cluster.[24] It thus provides the possibility of more precisely determining the actual extension of the rearrangements by analysing the wide α-globin cluster. Each probe target sequences are small between 50 to 70nt. In addition, the prerequisite of a ligation reaction for hybridised probes provides the opportunity to discriminate single nucleotide differences. Hence, the incorporation of one probe that is specifically designed for the detection of the CS mutation is possible using this method. Not only has advantages of being multiplex method, MLPA enable the detection of additional non-deletional abnormalities in one run, specifically HbCS which is the commonest non-deletional type in our population. Using the current Multiplex PCR (gap and ARMS), the simultaneous analysis is not likewise possible.

Moreover, in MLPA amplification is not directly on nucleic acids samples, but probes with identical end sequences added to the samples are amplified using one primer pair. Only ligated probes will be amplified, whereas non-ligated hemi-probes will not generate a signal; thus removal of unbound probe is not necessary. Therefore, MLPA protocol is extremely simple. As all fragments are amplified by using a single PCR primer pair, MLPA is more robust. Compared to multiplex PCR, it requires one pair of primers for each fragment to be amplified thus primers are present in large amounts during the reaction, resulting in various problems.

In order to get interpretable result, reference DNA samples are compulsory within each MLPA run. Minimum of 3 independent reference samples are recommended for a robust and reliable data analysis. During our analysis, we included 3 references DNA in each run. In 3 experiments, one of the 3 reference samples need to be excluded as a reference since the variability obtained was unacceptable. Therefore, only 2 references were used to performed analysis for those experiments. Although the overall probes that involved were fit for deletional and non-deletional types detected by Multiplex PCR, some of the results showed different expected probes ratio for the abnormalities as what being suggested in the MLPA protocol. One batch of experiment was repeated as all the reference DNA did not seem to be suitable as a reference sample resulting high variability which indeed unacceptable for interpretation. Liu *et al*[22] recommend using 2 to 4 normal reference samples during MLPA run. Overall, we suggest that if it happens more often that reference samples need to be excluded from the analysis, it may be an option to include at least 4 reference DNA in each experiment. This will allow exclusion of one reference sample at the same time minimum 3 references available to obtain reproducible and reliable results as suggested in the protocol. Performance of MLPA can be compromised by several factors.[18,19] Incomplete denaturation was identified as one of contributing factor. It was demonstrated in our experiment. The copy number changes in MLPA is based on relative changes in probe signals compare to reference samples where the target sequences of interest are assumed to have a normal copy number. Therefore, good reference amplification is required to ensure test reproducibility. We identified that prolongation of denaturation process to 10 minutes compare to 5 minutes as suggested in the MLPA general protocol provided better positive outcome. For the experiment with unacceptable references, the assay was initially run using 5 minutes denaturation step and showed unsatisfactory results. Repeated assay using 10 minutes denaturation produced successful analysis. Therefore, we applied this step throughout our MLPA analysis.

During this analysis, there were 2 discordant result from previous Multiplex PCR result

involving deletion type. Repeated analysis by both methods showed persistent findings respectively excluding false interpretation during the initial run. Retrospective analysis of clinical, haematological parameters and Hb analysis results were done. The first discordant sample was diagnosed as heterozygous αα/_α3.7, but MLPA findings revealed homozygous _α3.7/_α3.7. In addition, there was evidence of HBA gene conversion observed in the MLPA analysis. We examined the haematological indices, and it showed mildly reduced Hb level (10.0 g/dL) with MCV (74.6 fl) and MCH (23.4pg) value which was comparable to two gene deletion instead of one gene deletion as described by Azma *et al*.[12] and Ahmad *et al*.[5] There was no data on iron status in this patient to correlate with the effect of iron deficiency contributing to relatively lower Hb and red cell indices. However, finding of normal HbA2 level might exclude the concomitant effect of iron deficiency as low level of HbA2 is expected in a case of low iron. Sequencing analysis of both alpha globin genes subsequently confirmed the diagnosis of homozygous (_α3.7/_α3.7) and HBA1 gene conversion to HBA2 as detected by MLPA analysis.

The HBA1 and HBA2 are homologous except of few nucleotide differences. Due to the very high homology and close proximity of the HBA1 and HBA2, sequence exchange (gene conversion) can easily occur.[25] Alpha gene conversion may occur through a nonreciprocal transfer of information from HBA1 to HBA2 sequence or vice versa resulting in the production of two homologous recombination sequences, without any clinical consequences. Five probe pairs target locations with very small sequence differences between HBA1 and HBA2 are designed in MLPA analysis to detect the α-globin gene conversion which refer to African polymorphism in the analysis. In our case, HBA1 gene conversion to HBA2 has allowed α2-reverse primer to bind to the homologous sequences and amplify the α2 band, hence demonstrated as single gene deletion(αα/_α3.7) in multiplex PCR analysis. The discrepancy of initial phenotype-genotype findings was explained by the limitation of multiplex PCR in the presence of α-gene conversion. Our findings pointed the limitation of multiplex PCR in the present of this event. Sanger sequencing is sensitive for detecting the gene conversion in the α-globin gene cluster, but it is relatively expensive and also time-consuming. These findings highlight that using MLPA yielded a substantial increase in detection and is an appropriate method to characterise the unsolved α-globin gene rearrangement. In addition, it is a convenient alternative method to the more time-consuming, labour intensive and expensive methods given the fact that it enables detection of additional α-globin gene rearrangement in a single run. This functionality of MLPA was in agreement with findings described by Coloimo *et al*.[16] whom addressed MLPA as an appropriate option to the more time-consuming, labour intensive method such as SB or Gap PCR.

Second discordant case was homozygous_ α3.7/_α3.7, identified as a compound heterozygous state of _α3.7 with an uncharacterised two gene deletion that has similar pattern to Southeast Asian (_ _SEA) type. Phenotypically, this indicated HbH disease or thalassaemia intermedia as it involved 3 α-globin gene deletion. Therefore, a more severe phenotype was expected in this patient. This is a 28-year-old Chinese lady who presented at 38 weeks of pregnancy, accidentally noted to have moderate anaemia with Hb of 7.8g/dL requiring 2 units of packed cell transfusion. Red cell indices were in accordance to 3 α-globin gene deletion with MCV of 55.6fl and 15.7pg respectively.[5,12] Haemoglobin A2 was low (2.3%). There were hypochromic microcytic red cells and marked anisopoikilocytosis with the presence of fragmented cells, spherocytes, target cells and polychromatic cells on peripheral blood smear in keeping with the thalassaemia picture. The iron study was normal as evidenced by normal serum ferritin level. There was no detail documentation regarding physical findings of either hepatomegaly or splenomegaly which was expected to be present in HbH disease.[26] Gel electrophoresis did not show any fast band. Overall findings were in keeping with 3 genes rather than 2 genes involvement, consistent with MLPA findings. Although the exact breakpoint position and deletion lengths could not be determined at the time of the study, MLPA results showed three gene deletion that could not have been picked up by our multiplex PCR in the first place.

Malaysian Chinese have a higher incidence of heterozygous αα/_ _SEA deletion which was 51%.[5] Three α-gene deletions, _α3.7/_ _SEA is more common than _α3.7/_α3.7 among this ethnic with the incidence of 6.4% and 0.6%, respectively.[5] There are 25% risk of a pregnancy with Hb Bart's hydrops fetalis in a couple who are both α^0-thalassaemia carriers which is a fatal condition with significant

maternal complications. Given the facts of the high incidence of αα/_ _SEA among this ethnicity, it increases their predisposition of Hb Bart's hydrops fetalis pregnancy. In our study, even though we cannot comment on the details of the α^0 deleted region, identification of the compound deletion resulting in 3 gene involvement in this patient is crucial as it allows genetic counselling and antenatal diagnosis can be offered to the informed at-risk couples hence reproductive choice can be made accordingly. To gain more insight into the deletion present in this sample, further analysis is warranted and it would probably be best to do a family study. This may shed some more light on the actual deletion present in this sample.

To date, Hb CS mutation is the only non-deletional α-thalassaemia able to be detected by MLPA.[19] Our study demonstrated that MPLA detected all cases with Hb CS previously identified by multiplex PCR. In addition, none without the Hb CS mutation gave a false positive signal using the MLPA probe, indicating that it has 100% sensitivity and specificity in detecting the Hb CS mutation. This finding was comparable as described by Liu *et al*.[22] Compared to the deletional type, the Hb CS mutation-specific MLPA probe is primarily used to determine whether the mutation is present or absent. Therefore, we could not determine zygosity of Hb CS cases compared to multiplex PCR due to the limitation of the MLPA method. Determining whether the mutation is homozygous or heterozygous is possible, but this will require the use of and comparison to positive control samples with a known copy number status of the mutation. Inclusion of such known positive samples will then allow inter-sample normalisation, thereby facilitating the determination of the copy number for the CS mutation. However, this was not performed in this analysis.

Hb CS is the most prevalent non-deletional type of α-thalassemia in Asian. This finding was also observed in our population, and it was the third most frequent α-thalassemia determinant among Malays and Sabahans in Malaysia.[22] The diagnosis can be suspected by the presence of Hb CS peak on CE or HPLC analysis in addition to red cell parameters. Since MLPA included a probe dedicated to HbCS detection as part of multiple deletional probes, it enables additional detection of this high prevalent non-deletional type in one run thus giving the advantage to limit analysis in a single run in an endemic setting as our population. On the other hand, zygosity of Hb CS can be possibly suspected based on clinical presentation, severity of anaemia and percentage of Hb CS by Hb analysis.[26] Homozygotes for this variant are usually anaemic (mean Hb 10g/dL) with low MCH (mean 26pg) but normal or low normal MCV (mean 88fl). Percentage of Hb CS ranges between 2-11% and Hb Bart is present between 1-3%. Hence, strategised analysis can be offered in which only those who were suspected to have homozygous state may further subject for PCR ARMS analysis. Even though zygosity state cannot be determined by MLPA analysis, incorporating family study, clinical and haematological findings in the decision for subsequent analysis will help to prevent unnecessary analysis, thus reduce the workload burden and ensure laboratory cost effectiveness particularly in resource-limited state.

In conclusion, this study showed the ability of MLPA assay to effectively identified gene mutations in α-thalassaemia. Both MLPA and multiplex PCR showed reliable and accurate technique, however by analysing the wide α-globin cluster, MLPA seem to be more precise in determining the actual extension of the rearrangements in one single experiment. The robustness, simplicity with additional specificity offered by the ligation step make MLPA to be favourable diagnostic method for the detection and characterisation of deletion/duplication involved α-globin gene. Using only MLPA as a complementary method to identify α-thalassaemia carriers in our patients yielded a substantial increase in the detection of carriers and hence provided more accurate information to guide counselling. Our findings support that MLPA method provides simple, sensitive and cost-effective approach for α-thalassaemia, particularly in ethnically diverse populations like us. We propose that incorporating MLPA as a second-tier method in routine laboratory practice in high-prevalence regions such as ours, where haematological evidence of α-thalassaemia trait may increase the detection rate, thereby allowing more options for prenatal diagnosis. Unresolved cases may be further subjected to additional methods such as Sanger sequencing. Incorporating MLPA will benefit in improving the panel of detection for the diagnosis of α-thalassaemia comprehensively hence hopefully helps our aim to ameliorate the screening and management of α-thalassaemia as a challenging public health concern in our population.

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