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Allele-specific PCR for a cost-effective & time-efficient diagnostic screening of spinal muscular atrophy

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***Background & objectives*: Genetic diagnosis of spinal muscular atrophy (SMA) is complicated by the presence of *SMN2* gene as majority of SMA patients show absence or deletion of *SMN1* gene. PCR may amplify both the genes non selectively in presence of high amount of DNA. We evaluated whether allele-specific PCR for diagnostic screening of SMA is reliable in the presence of high amount of genomic DNA, which is commonly used when performing diagnostic screening using restriction enzymes.**

***Methods*: A total of 126 blood DNA samples were tested in amounts ranging 80-200 ng, referred for the genetic diagnosis of SMA using both conventional PCR-RFLP and allele-specific PCR.**

***Results*: The results from both methods showed agreement. Further, allele-specific PCR was found to be a time-efficient and cost-effective method.**

***Interpretation & conclusions*: Our study demonstrated the accuracy of our allele-specific PCR and the results were comparable compatible with that of PCR-RFLP, indicating its practical application in SMA diagnostic screening.**

Key words Allele-specific PCR - PCR-RFLP - SMA diagnostic screening - *SMN1* - spinal muscular atrophy

Conventional PCR-RFLP for genetic diagnosis of spinal muscular atrophy (SMA)[1] has been considered time consuming and expensive. It requires restriction enzyme (RE) digestion which uses a consi derably high amount of PCR product. If the amount of the PCR product is higher than necessary, this may lead to partial RE digestion resulting in the appearance of undigested PCR product on gel electrophoresis (*i.e.* false-negative results).

The responsible genes for SMA are *Survival Motor Neuron* (*SMN*) genes. The *SMN* genes consist of two highly identical genes; *SMN1* (telomeric *SMN*) and *SMN2* (centromeric *SMN*) which share over 99.8 per cent sequence homology over a 30 kb segment. *SMN1* and *SMN2* can be distinguished by only five nucleotides differences located in intron 6, exon 7, intron 7 and exon 8[2]. Ninety five per cent of SMA patients showed

an absence of *SMN1* gene due to either deletion or conversion, thus demonstrating that *SMN1*, not *SMN2,* is the SMA-causing gene[3]. Thus, genetic diagnosis of SMA (*i.e.* detection of *SMN1* deletion) was complicated by the presence of *SMN2* because PCR may amplify the genes unselectively, especially in the presence of high amount of genomic DNA, while all patients carry *SMN2* gene. Allele-specific PCR for the genetic diagnosis of SMA has been described elsewhere from as early as 1999[4-9]. However, to the best of our knowledge studies involving high amount of DNA, the amount of which is routinely used for genetic diagnosis, provided conflicting results regarding chances of *SMN2* mis-amplification. In addition, no study has been done to evaluate the cost-effectiveness and time-efficiency of this method over conventional PCR-RFLP. Using relatively larger sample size, we studied the reliability of allele-specific PCR by comparing the test results against conventional PCR-RFLP using high amount of genomic DNA.

Material & Methods

This study was carried out from 2003 to 2008 in the Department of Paediatric, School of Medical Sciences, Universiti Sains Malaysia, Kelantan. A total of 126 patients were randomly selected from the patients sent for SMA genetic diagnosis (Table I). Whole blood (3-5 ml) was collected from patients. Informed consent was taken prior to blood taking. The study protocol was approved by the Research Ethics Committee (Human) of the Universiti Sains Malaysia. Sample size was calculated using single proportion formula. Genomic DNA was extracted using commercially available kit (GeneAll Biotechnology Co. Ltd., Korea).

PCR-RFLP (Method A): All samples were analyzed twice, each using two different methods, method A and method B. Method A refers to the PCR-RFLP as previously described[1]. This method consisted of two steps, PCR amplification and enzyme digestion which used *Dra1* restriction enzyme for exon 7 *SMN*.

Allele-specific PCR (Method B): Method B refers to allele-specific PCR using primer pairs described previously[4], telSMNex7forw 5'-TTTATTTTCCTTACAGGGTTT*C*-3' and telSMNint7rev 5'-GTGAAAGTATGTTTCTTCCAC***g****T*A-3'. Italic uppercase characters indicate the position of nucleotide difference between *SMN1* and *SMN2*, while lowercase character indicates the position of a deliberate mismatch. The primers specifically amplify exon 7 of *SMN1*, not *SMN2*. This method consisted of only one PCR amplification step. Positive or negative interpretation was determined visually on agarose gel electrophoresis by the absence or presence of the *SMN1* exon 7, respectively. To monitor the efficiency of PCR amplification, a housekeeping gene (β-globin) was used as a reference gene, with the primers 5-ACCTCACCCTGTGGAGCCAC-3 and 5-CTCACCACCAACTTCATCCAAG-3. One

Table I. Clinical features of the patients

Clinical features			Number of patients	Total
Clinically SMA	Sex	Male	25	
		Female	38	63
	Age on diagnosis (Month)	0-6	11	
		6-18	13	
		>18	39	63
	Type of SMA	I	31	
		II	23	
		III	9	63
	Tongue Fasciculation	+ve	35	
		-ve	20	55*
	EMG	+ve	20	
		-ve	1	21*
Clinically Not SMA	Sex	Male	30	
		Female	33	63
	Age on diagnosis (Month)	0-6	20	
		6-18	19	
		>18	22	61*

EMG; electromyography, *Data not available for some patients

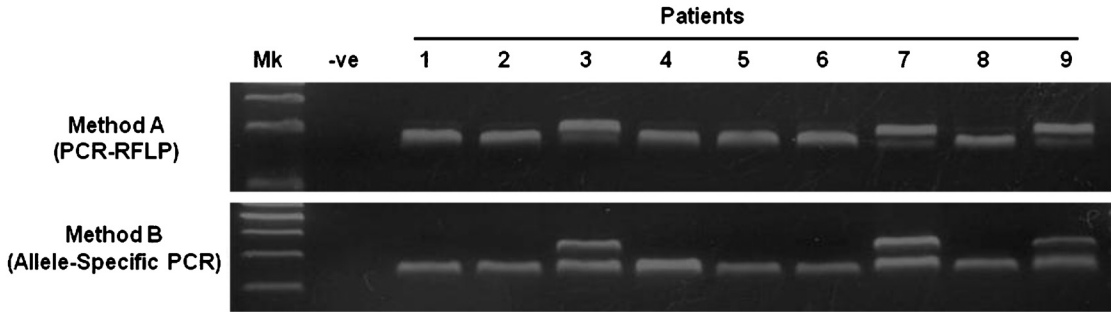

Fig. *SMN1* (exon 7) deletion analysis using method (A) and (B). In method (A), deletion or non-deletion is indicated by the absence or presence of the first band (188 bp). The second band (164 bp) shows the presence of *SMN2* (exon 7). In method (B), deletion or non-deletion is indicated by the absence or presence of the first band (307 bp). The second band (240 bp) indicated the presence of reference gene (β-globin). Molecular markers were electrophoresed in the "Mk" lane and a control PCR product in the "–ve" lane. Patients 1, 2, 4, 5, 6, 8 show deletion of *SMN1*. Patients 3, 7, 9 show non-deletion of *SMN1*.

reaction of 20 µl of PCR mixture contained 80 - 200 ng of genomic DNA, 0.4 µl of 10 mM dNTPs, 1.2 µl of 25 mM $MgCl_2$, 4.0 µl of 5x PCR buffer, 0.75U *Taq* DNA polymerase (Promega Corporation, Madison, USA) and 1.5 µl of each 10 pmol of an allele specific primer pairs and 0.5 µl of each 5 pmol of an internal control primer pairs (Sigma-Proligo, The Woodlands, TX, USA). The PCR cycles included an initial denaturation at 94°C for 7 min, followed by 33 cycles of 94°C for 1 min, 59.7°C for 1 min, 72°C 1 min before a final extension at 72°C for 7 min. The PCR product was then directly visualized under the UV light, using 2 per cent agarose gel. PCR product of *SMN1* was visualized at the corresponding size of 307 bp, while that of β-globin was at the size of 240 bp.

Cost and time evaluation: The cost and time for both methods were compared to evaluate the cost-effectiveness and time-efficiency of allele-specific PCR over PCR-RFLP.

Results & Discussion

Of the 126 samples tested, 54 (43%) were found to have *SMN1* exon 7 deletions. The findings (Fig.) from both methods were in complete agreement, suggesting that both methods performed with the same reliability. Evaluation of time-efficiency for both methods showed that method B was five times more rapid than method A. Evaluation on the cost-effectiveness showed that method B was more cost-effective (68%) compared to method A (Table II).

Table II. Comparison of time-efficiency and cost-effectiveness between PCR-RFLP and allele-specific PCR in SMA genetic diagnosis

Items	PCR-RFLP (A) (min)		Allele-specific PCR (B) (min)	
A. Time-efficiency comparison				
1. PCR mixture preparation	30		30	
2. PCR thermocycling	150		150	
3. Gel electrophoresis I	40		40	
4. PCR-RFLP preparation	10		-	
5. Incubation	960		-	
6. Gel electrophoresis II	40		-	
Total	1230		220	
B. Cost-effectiveness comparison	PCR-RFLP		Allele-specific PCR	
	(INR)	(US$)	(INR)	(US$)
1. PCR reagents	949	16.9	949	16.9
2. Restriction enzyme	2336	41.6	-	-
3. Gel electrophoresis	190	3.4	95	1.7
4. Consumables	219	3.9	95	1.7
Total	3694	65.8	1139	20.3

*based on US$ 1 = INR 56.15

Table III. Summary of previous studis on diagnosis of SMA using allele specific PCR

Author	Primers	Technique	Outcome
Feldkötter *et al*[4]	Forward (c.840C>T) Reverse (c.888+214A>G)	Quantitative real-time PCR of *SMN1* using LightCycler instrument (Roche Diagnostics)	*SMN1*-specific amplification from 11.25ng of genomic DNA.
Xu *et al*[8]	Specific reverse primer (c.840C>T)	PCR	Lower analytical sensitivity/specificity, especially with lower (≤ 0.1 pg) and higher (≥ 1 pg) DNA amount.
Moutou *et al*[5]	Specific reverse primer (c.840C>T)	PCR with fluorescent technology	*SMN1*-specific amplification from up to 1ng of genomic DNA.
Zeng *et al*[9]	Specific reverse primer (c.840C>T)	PCR	*SMN1*-specific amplification from 50ng of genomic DNA.
Ravard-Goulvestre *et al*[6]	Specific reverse primer (c.840C>T)	PCR with fluorescent technology	*SMN1*-specific amplification from up to 700ng of genomic DNA.
Simsek *et al*[7]	Specific forward primer (c.840C>T)	PCR	Successfully amplified *SMN1* by using 100ng of DNA amount and the results were comparable with the PCR-RFLP.

We have been using the primers described by Feldkötter *et al*[4] for gene-specific copy number analysis of *SMN1* and *SMN2* as a diagnostic procedure in our laboratory and found that our results were consistent[10,11]. However, the experiments were done with relatively low amount of DNA, by which the chance for *SMN2* mis-amplification was very small.

In this study, 54 samples showed deletion in *SMN1* gene exon 7. Among the remaining 72 patients without *SMN1* deletion, nine were categorized as clinically SMA (Table I). Patients without apparent deletion of *SMN1* may not be excluded from the SMA diagnosis since possibility remains that they might carry point mutation which can only be identified through DNA sequencing.

Our analyses showed that allele-specific PCR was a time-efficient and cost-effective method. It may also reduce the risk for experimental errors since it involves fewer steps.

The main differences of the method described here compared to other similar methods is the design of the primers, the usage of conventional PCR method and the usage of a relatively higher amount of genomic DNA than the amount which is routinely used for SMA genetic diagnosis. Using primer pairs described by Feldkotter *et al*[4]. We could specifically amplify *SMN1* in the existence of relatively high DNA amount (80 - 200ng), without mis-amplifying *SMN2*, thus enabling the use of the method for routine genetic testing of SMA. However, the use of higher amount of DNA in this study has not provided evidence if the test is still reliable in the presence of much lower DNA amount as described elsewhere[5,8]. This could be a major hurdle for applications such as preimplantation genetic diagnosis (PGD).

The primers were selected because these fulfilled the criteria for highly-efficient allele-specific amplification for utilizing two nucleotides differences between *SMN1* and *SMN2* in exon 7 (c.840C>T) and intron 7 (c.888+214A>G) and a deliberate primer mismatch simultaneously in one PCR reaction. *SMN1* carries the C and A in its exon 7 and intron 7, respectively. The forward primer (telSMNex7forw) incorporated the C at its first nucleotide at the 3' end, while the reverse (telSMNint7rev) combined an incorporation of A at its 2nd nucleotide before the 3' end and a deliberate mismatch at its 3rd nucleotide before the 3'end (G instead of A). Newton *et al*[12] showed that a deliberate mismatch near to the primer's 3'end increased its amplification specificity. Therefore, the primer pair described by Feldkotter *et al*[4] contained three characteristics, a specific forward, a specific reverse and a deliberate mismatch.

Similarly, specific primers may be designed to detect the presence or absence of *SMN1* exon 8. However, we concentrated only on exon 7 of the *SMN1* gene in this study because it is the only region with the clinical significance for the diagnostic screening of SMA[13]. The summary of the previous studies were shown in Table III.

In conclusion, our study demonstrated the reliability of allele-specific PCR for diagnostic screening of SMA. The accuracy of this method was comparable with that of PCR-RFLP, and it was cost-

effective. Thus, it can be applied to routine diagnostic screening of SMA.

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References

1. van der Steege G, Grootscholten PM, van der Vlies P, Draaijers TG, Osinga J, Cobben JM, *et al*. PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. *Lancet* 1995; *345* : 985-6.
2. Bürglen L, Lefebvre S, Clermont O, Burlet P, Viollet L, Cruaud C, *et al*. Structure and organization of the human survival motor neurone (*SMN*) gene. *Genomics* 1996; *32* : 479-82.
3. Lefebvre S, Bürglen L, Reboullet S, Clermont O, Burlet P, Viollet L, *et al*. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 1995; *80* : 155-65.
4. Feldkötter M, Schwarzer V, Wirth R, Wienker T F, Wirth B. Quantitative analyses of *SMN1* and *SMN2* based on realtime light-cycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am J Hum Genet* 2002; 70 : 358-68.
5. Moutou C, Gardes N, Rongières C, Ohl J, Bettahar-Lebugle K, Wittemer C, *et al*. Allele-specific amplification for pre-implantation genetic diagnosis (PGD) of spinal muscular atrophy. *Prenat Diagn* 2001; *21* : 498-503.
6. Ravard-Goulvestre C , Boucly C, Mathieu B, Van Amerongen G, Viollet L, Estournet B, *et al*. Allele-specific amplification for the diagnosis of autosomal recessive spinal muscular atrophy. *Clin Chem Lab Med* 1999; *37* : 133-5.
7. Simsek M, Al-Bulushi T, Shanmugakonar M, Al-Barwani HS, Bayoumi R. Allele-specific amplification of exon 7 in the survival motor neuron (SMN) genes for molecular diagnosis of spinal muscular atrophy. *Genet Test* 2003; *7* : 325-7.
8. Xu R, Ogino S, Lip V, Fang H, Wu BL. Comparison of PCR-RFLP with allele-specific PCR in genetic testing for spinal muscular atrophy. *Genet Test* 2003; *7* : 277-81.
9. Zeng J, Lan F, Deng X, Ke L, Tu X, Huang L, *et al*. Evaluation of an in-house protocol for prenatal molecular diagnosis of SMA in Chinese. *Clin Chim Acta* 2008; *398* : 78-81.
10. Tran VK, Sasongko TH, Hong DD, Hoan NT, Dung VC, Lee MJ, *et al*. *SMN2* and NAIP gene dosages in Vietnamese patients with spinal muscular atrophy. *Pediatr Int* 2008; *50* : 346-51.
11. Watihayati MS, Fatemeh H, Marini M, Atif AB, Zahiruddin WM, Sasongko TH, *et al*. Combination of SMN2 copy number and NAIP deletion predicts severity in spinal muscular atrophy. *Brain Dev* 2009; *31* : 42-5.
12. Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, *et al*. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acid Res* 1989; *17* : 2503-16.
13. Sasongko TH, Gunadi, Zilfalil BA, Zabidi-Hussin ZAMH. SMN1 exon 7 deletion analysis may be necessary and sufficient for the diagnosis of spinal muscular atrophy. *J Neurogenet* 2011; *25* : 15-6.

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