Kobe J. Med. Sci., Vol. 63, No. 2, pp. E37-E40, 2017

New, Improved Version of the mCOP-PCR Screening System for Detection of Spinal Muscular Atrophy Gene (*SMN1*) Deletion

MASAKAZU SHINOHARA[1], MAWADDAH AR ROCHMAH[1], KENTA NAKANISHI[1], NUR IMMA FATIMAH HARAHAP[1], EMMA TABE EKO NIBA[1], TOSHIO SAITO[2], KAYOKO SAITO[3], ATSUKO TAKEUCHI[4], YOSHIHIRO BOUIKE[5], and HISAHIDE NISHIO[1*]

[1] *Department of Community Medicine and Social Healthcare Science, Kobe University Graduate School of Medicine, Kobe, Japan;*

[2] *Division of Child Neurology, Department of Neurology, National Hospital Organization Toneyama National Hospital, Toneyama, Japan;*

[3] *Institute of Medical Genetics, Tokyo Women's Medical University, Tokyo, Japan;*

[4] *Kobe Pharmaceutical University, Kobe, Japan;*

[5] *Faculty of Nutrition, Kobe Gakuin University, Kobe, Japan*

Received 10 April 2017/ Accepted 1 May 2017

Keywords: spinal muscular atrophy, *SMN1*, *SMN2*, mCOP-PCR, targeted pre-amplification

BACKGROUND: Spinal muscular atrophy (SMA) is a frequent autosomal recessive disorder, characterized by lower motor neuron loss in the spinal cord. More than 95% of SMA patients show homozygous survival motor neuron 1 (*SMN1*) deletion. We previously developed a screening system for *SMN1* deletion based on a modified competitive oligonucleotide priming-PCR (mCOP-PCR) technique. However, non-specific amplification products were observed with mCOP-PCR, which might lead to erroneous interpretation of the screening results. AIM: To establish an improved version of the mCOP-PCR screening system without non-specific amplification. METHODS: DNA samples were assayed using a new version of the mCOP-PCR screening system. DNA samples had already been genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP), showing the presence or absence of *SMN1* exon 7. The new mCOP-PCR method contained a targeted pre-amplification step of the region, including an *SMN1*-specific nucleotide, prior to the mCOP-PCR step. mCOP-PCR products were electrophoresed on agarose gels. RESULTS: No non-specific amplification products were detected in electrophoresis gels with the new mCOP-PCR screening system. CONCLUSION: An additional targeted pre-amplification step eliminated non-specific amplification from mCOP-PCR screening.

INTRODUCTION

Spinal muscular atrophy (SMA) is a frequent autosomal recessive disorder with an incidence of 1/10,000 live births [11]. The SMA locus has been mapped to chromosome 5q13 using linkage analysis [1,9], and is divided into telomeric and centromeric parts because of the presence of a large inverted duplication in the chromosome [7,8].

In 1995, the survival motor neuron gene (*SMN*) was reported as a candidate gene for SMA [7]. *SMN* exists in two nearly identical copies, *SMN1* (the telomeric copy) and *SMN2* (the centromeric copy) [7]. *SMN1* is present in all healthy individuals, but absent from more than 95% of SMA patients and deleteriously mutated in the remaining patients [7]. The gene product, SMN, may be an essential protein in distinct cellular functions including heterogeneous nuclear ribonucleoprotein biogenesis (a ubiquitous function) and axonal transport (a motor neuron-specific function) [10]. Thus, *SMN1* is now considered as an SMA-causing gene.

On the other hand, *SMN2* was previously considered to be dispensable because approximately 5% of normal individuals do not carry the gene [7]. However, *SMN2* also expresses SMN [7], albeit at considerably lower levels than *SMN1*. Thus, SMA is caused by low SMN levels rather than the complete absence of SMN [2,12]. It was shown that a high copy number of *SMN2* can partially compensate for the lack of *SMN1* [3,15]. Therefore, *SMN2* is also now considered to be an SMA-modifying gene.

Complete loss of both *SMN* genes may cause embryonic lethality, therefore all SMA patients with homozygous *SMN1* deletions carry at least one copy of the *SMN2* gene [5,13]. *SMN2* is highly homologous to *SMN1*, with only five nucleotide differences between them [7]. However, high similarity with *SMN2* hampers detection of homozygous *SMN1* deletions by conventional PCR methods.

Phone: +81-78-382-5540 Fax: +81-78-382-5559 E-mail: nishio@med.kobe-u.ac.jp

To address this problem, we developed a screening system for homozygous *SMN1* deletion based on a competitive oligonucleotide priming-PCR (COP-PCR) technique, which separately amplifies *SMN1* exon 7 and *SMN2* exon 7 [6]. We previously used the term "COP-PCR" for this method, in spite of some modifications added to the original COP-PCR method [4]. We have decided now to use the term "modified competitive oligonucleotide priming-PCR (mCOP-PCR)" in this study, and hereafter, to refer to the method.

Although mCOP-PCR separately amplified *SMN1* exon 7 and *SMN2* exon 7, we were also aware that non-specific amplification products of unexpected sizes often appeared in mCOP-PCR. It might lead to erroneous interpretation of screening results. In this study, we established a new, improved mCOP-PCR screening system, without non-specific amplification, to avoid any ambiguity of the results.

MATERIAL AND METHODS

Patient and control samples

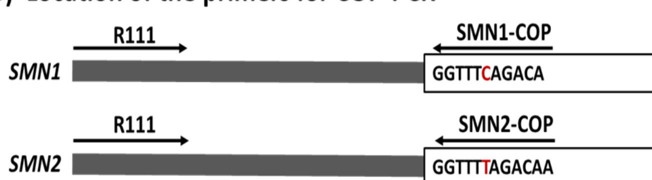
Two DNA samples were assayed from each of three individuals (two controls and one SMA patient). One DNA sample was extracted from fresh whole blood by the SepaGene agglutination partition method (EIDIA, Tokyo, Japan). The other sample was extracted from a dried blood spot on filter paper by the method of Kato *et al*. [6]. The individuals had already been genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP), showing the presence or absence of *SMN1* exon 7. Prior to analysis, informed consent was obtained from study participants. The study was approved by the Ethics Committee of Kobe University Graduate School of Medicine.

Outline of the new mCOP-PCR screening system

The new mCOP-PCR screening system included the following steps: (1) preparation of DNA samples; (2) targeted pre-amplification of sequence containing *SMN1*/*SMN2* exon 7 by conventional PCR to secure the quality and quantity of each DNA sample; and (3) gene-specific amplification of *SMN1*/*SMN2* exon 7 using mCOP-PCR. PCR products were electrophoresed on 4% agarose gels. The primer locations and sequences used in each PCR step are shown in Figure 1.

(A) Locations of the primers for targeted pre-amplification

(B) Location of the primers for COP-PCR



(C) Primer sequences

R111 [1995 Lefebvre]: 5'-AGA CTA TCA ACT TAA TTT CTG ATC A-3'
X7-Dra [1995 Steege]: 5'-CCT TCC TTC TTT TTG ATT TTG TTT -3'
SMN1-COP [2014 Kato]: 5'-TGT CTG AAA CC-3'
SMN2-COP [2014 Kato]: 5'-TTG TCT AAA ACC-3'

Figure 1. Locations and sequences of the primers.
Black bars in (A) and (B) represent intron 6 of survival motor neuron 1 (*SMN1*) and survival motor neuron 2 (*SMN2*). Boxes in (A) and (B) represent exon 7 of both genes. Letters in the boxes represent the sequence flanking gene-specific nucleotides (red letters). "C/T" indicates the presence of both gene-specific nucleotides.
(A) Location of primers for targeted pre-amplification.
(B) Location of primers for competitive oligonucleotide priming-PCR (COP-PCR).
(C) Primer sequences. The X7-Dra primer has a mismatched nucleotide (blue letter) to the G nucleotide, which is two nucleotides away from the gene-specific nucleotide. Red letters in SMN1-COP and SMN2-COP primers represent the matched nucleotide to the gene-specific nucleotide.

Targeted pre-amplification

Targeted pre-amplification of the sequence containing *SMN1/2* exon 7 was performed by conventional PCR using GeneAmp® PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Foster City, CA, USA). Two µl of template solution (equivalent to 100 ng DNA from fresh blood or 200–300 ng DNA from a dried blood spot) was directly added to PCR mixture (total volume, 28 µl) containing 1× PCR buffer, 2 mM $MgCl_2$, 0.1 mM of each dNTP, 0.3 µM of each primer (R111 and X7-Dra), and 1.0 U FastStart Taq DNA polymerase (Roche Applied Science, Mannheim, Germany). The PCR conditions were: (1) initial denaturation at 94°C for 7 min; (2) 30 or 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min (30 cycles for fresh blood DNA and 40 cycles for dried blood spot DNA); (3) additional extension at 72°C for 7 min; and (4) hold at 10°C. Afterwards, an aliquot of pre-amplified product was electrophoresed on a 4% agarose gel in 1× TBE buffer, and visualized by Midori-Green staining (Nippon Genetics, Tokyo, Japan).

Gene-specific amplification of *SMN1* and *SMN2* exon 7 by mCOP-PCR

mCOP-PCR was performed using the GeneAmp® PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific). After 100-fold dilution of pre-amplified product, 2 µl diluted solution was added to PCR mixture (total volume, 28 µl) containing 1× PCR buffer, 2 mM $MgCl_2$, 0.1 mM of each dNTP, 0.3 µM of common forward primer (R111), 3 µM of gene-specific reverse primer (SMN1-COP or SMN2-COP), and 1.0 U FastStart Taq DNA polymerase. The PCR conditions were: (1) initial denaturation at 94°C for 7 min; (2) 20 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, and extension at 72°C for 1 min; (3) additional extension at 72°C for 7 min; and (4) hold at 10°C. Afterwards, an aliquot of each PCR product was electrophoresed on a 4% agarose gel in 1× TBE buffer, and visualized by Midori-Green staining.

RESULTS

We performed the old and new versions of mCOP-PCR analysis using 100 ng fresh blood DNA or 200–300 ng dried blood spot DNA. The old version included 30 or 40 cycles of mCOP-PCR (30 cycles for fresh blood DNA and 40 cycles for dried blood spot DNA) without targeted pre-amplification, while the new version included 30 or 40 cycles of conventional PCR for targeted pre-amplification (30 cycles for fresh blood DNA and 40 cycles for dried blood spot DNA) followed by 20 cycles of mCOP-PCR.

We succeeded in gene-specific amplification of *SMN1* exon 7 and/or *SMN2* exon 7 from all three individuals using fresh blood DNA and dried blood spot DNA, with or without the targeted pre-amplification step (Figure 2B). The target amplification products were easily identified by their size (or band location in agarose gels after electrophoresis). PCR product sizes of *SMN1* exon 7 and *SMN2* exon 7 were 168 bp and 169 bp, respectively. The results of mCOP-PCR analysis were fully compatible with genotyping analysis data obtained by PCR-RFLP.

Without the targeted pre-amplification step, non-specific amplification products were observed in fresh blood DNA, with many detected in dried blood spot DNA samples. Indeed, the number and intensity of non-specific amplification products were so high in dried blood spot DNA samples that the reliability of any potential results would be hampered. In contrast, when mCOP-PCR was performed with targeted pre-amplification, these non-specific amplification products were absent in both fresh blood and dried blood spot DNA samples.

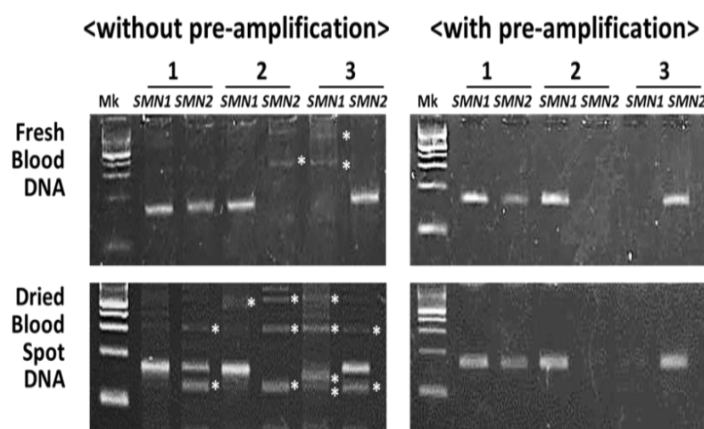


Figure 2. Selective detection of *SMN1* and *SMN2*.
Genotyping by PCR-restriction fragment length polymorphism (PCR-RFLP). Individual 1 is a healthy control carrying both survival motor neuron 1 (*SMN1*) and survival motor neuron 2 (*SMN2*). Individual 2 is a healthy control carrying only *SMN1*, but not *SMN2*. Individual 3 comes from a spinal muscular atrophy (SMA) patient not carrying *SMN1*, but retaining *SMN2*.
Selective amplification of *SMN1* and *SMN2* by modified competitive oligonucleotide priming-PCR (mCOP-PCR). When mCOP-PCR was performed without targeted pre-amplification, non-specific amplification products (asterisks) were obtained in fresh blood samples, with many (asterisks) detected in dried blood spot DNA. In contrast, with targeted pre-amplification, no non-specific amplification products were observed in either DNA samples. Mk represents the markers of 100 bp DNA ladder; the lowest band shows a 100 bp marker.

DISCUSSION

Here, we compared a new version of mCOP-PCR with a targeted pre-amplification step to the old version without a targeted pre-amplification step. The old version of mCOP-PCR screening system has been described previously [6]. In comparison, the new version simply incorporates a targeted pre-amplification step. Targeted pre-amplification was so efficient that non-specific amplification was eliminated. Consequently, interpretation of mCOP-PCR results becomes simplified and straightforward.

Frequent appearance of non-specific amplification products in the old mCOP-PCR screening system may be related to DNA quality. This is supported by the fact that non-specific amplification products were more abundant in dried blood spot DNA samples than fresh blood DNA samples. The quality of DNA extracted from dried blood spots on filter paper is poorer than DNA extracted from fresh blood, and this disparity becomes larger with a longer storage period. Addition of a targeted pre-amplification step prior to mCOP-PCR may overcome the poor quality of dried blood spot DNA.

The new mCOP-PCR screening system takes longer time compared with the old version (~ 4 hours vs. ~ 3 hours). However, the results obtained with the new version are unambiguous. In addition, the primers used in the targeted pre-amplification step, R111 and X7-Dra, are also used for PCR-RFLP analysis [14]. Accordingly, the pre-amplified products can be used directly for PCR-RFLP. The results of mCOP-PCR can be checked by PCR-RFLP, although an overnight incubation at 37°C is needed.

In conclusion, we have established an improved version of the mCOP-PCR screening system without non-specific amplification. An additional targeted pre-amplification step prior to mCOP-PCR succeeded in eliminating non-specific amplification in the mCOP-PCR screening system. Finally, it should be noted that although our screening system described here is very useful for the detection of *SMN1*-deletion, it is not for the detection of an intra-genic mutation in *SMN1*. The absence of *SMN1* deletion does not neglect the possibility of SMA caused by an intragenic mutation in *SMN1*.

ACKNOWLEDGMENTS

This research was supported in part by the Practical Research Project for Rare/Intractable Diseases from the Japan Agency for Medical Research and Development, Grant No. 16ek0109086h0002 (title “Practical study for multicenter cooperative and investigator initiated clinical trial using valproic acid in childhood onset spinal muscular atrophy”).

REFERENCES

1. **Brzustowicz, L.M., Lehner, T., Castilla, L.H., et al.** 1990. Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3. Nature **344**: 540-541.
2. **Burghes, A.H. and Beattie, C.E.** 2009. Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? Nat Rev Neurosci. **10**: 597-609.
3. **Feldkötter, M., Schwarzer, V., Wirth, R., et al.** 2002. Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR; fast and highly reliable carrietest and prediction of spinal muscular atrophy. Am J Hum Genet **70**: 358-368.
4. **Gibbs, R.A., Nguyen, P., and Caskey, C.T.** 1989. Detection of single DNA base differences by competitive oligonucleotide priming. Nucleic Acids Res. **17**:2437-48
5. **Hsieh-Li, H.M., Chang, J.G., Jong, Y.J., et al.** 2000. A mouse model for spinal muscular atrophy. Nat Genet. **24**: 66-70.
6. **Kato, N., Sa'Adah, N., Ar Rochmah, M., et al.** SMA screening system using dried blood spots on filter paper: application of COP-PCR to the SMN1 deletion test. 2014. Kobe J Med Sci. **60**: E78-85.
7. **Lefebvre, S., Bürglen, L., Reboullet, S., et al.** 1995. Identification and characterization of a Spinal Muscular Atrophy-determining gene. Cell **80**: 155-165.
8. **Lewin, B.** 1995. Genes for SMA: Multum in Parvo. Cell **80**: 1-5.
9. **Melki, J., Abdelhak, S., Sheth, P., et al.** 1990. Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q. Nature **344**: 767-768.
10. **Nurputra, D.K., Lai, P.S., Harahap, N.I., et al**. 2013. Spinal Muscular Atrophy: From Gene Discovery to Clinical Trials. Ann Hum Genet. **77**: 435-463.
11. **Pearn, J.** 1978. Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. J Med Genet. **15**: 409-413.
12. **Prior, T.W.** 2011. Perspectives and diagnostic considerations in spinal muscular atrophy. Genet Med. **12**: 145-152.
13. **Schrank, B., Götz, R., Gunnersen, J.M., et al.** 1997. Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. Proc Natl Acad Sci. **94**:9920-9925.
14. **Van der Steege, G., Grootscholten, P.M., Van der Vlies, P., et al.** 1995. PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. The Lancet **345**: 985-986.
15. **Velasco, E., Valero, C., Valero, A., et al**. 1996. Molecular analysis of the SMN and NAIP genes in Spanish spinal muscular atrophy (SMA) families and correlation between number of copies of cBCD541 and SMA phenotype. Hum Mol Genet. **5**: 257-263.