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SMA Screening System Using Dried Blood Spots on Filter Paper: Application of COP-PCR to the *SMN1* Deletion Test

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BACKGROUND: Spinal muscular atrophy (SMA) is a common neuromuscular disorder caused by mutations in *SMN1*. More than 95% of SMA patients carry homozygous *SMN1* deletions. Thus, the *SMN1* deletion test should be performed initially as part of the diagnostic process. However, *SMN2*, a highly homologous gene, hampers detection of *SMN1* deletion. To differentiate between *SMN1* and *SMN2*, many analysis methods have been developed yet they are not all available worldwide. AIM: To establish a simple but accurate *SMN1*-deletion detection system that can be used worldwide. METHODS: Fifty DNA samples (29 SMA patients and 21 controls) from dried blood spots (DBS) on filter paper were assayed. All participants had previously been screened for SMA by PCR-restriction fragment length polymorphism (PCR-RFLP) using DNA extracted from freshly collected blood. DNA was extracted from DBS that had been stored at room temperature (20–25°C) for between 1 and 8 years. Competitive oligonucleotide priming-PCR (COP-PCR) was performed to distinguish *SMN1* and *SMN2* exon7. RESULTS: DNA yield from an 11-mm diameter DBS circle was 21,171 ± 7,485 ng (mean ± SD), with an 260/280 OD ratio from 1.49 to 2.1(mean ± SD; 1.67 ±0.13). Nucleotide sequencing confirmed gene-specific amplification of *SMN1* and *SMN2* by COP-PCR. *SMN1* and *SMN2* COP-PCR results are completely consistent with those obtained by PCR-RFLP. CONCLUSION: We have combined DNA extraction from DBS on filter paper with COP-PCR that specifically detects *SMN1* and *SMN2*, establishing a new *SMN1*-deletion detection system with practical application worldwide.

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INTRODUCTION

Spinal muscular atrophy (SMA) causes general muscle weakness because of a loss of lower motor neurons in the spinal cord. SMA is a common autosomal recessive disorder, with an incidence of 1/10,000 live births [24]. SMA is classified into three subtypes that depends on age of disease onset and achievement of motor milestones [19]: type 1 (severe form; onset age of 0–6 months, unable to sit unaided), type 2 (intermediate form; onset age of <18 months, unable to stand or walk unaided), and type 3 (mild form; onset age of >18 months, able to stand or walk unaided). Additionally, two other forms have been reported, SMA type 0 is the most severe and has a prenatal onset, while SMA type 4 is mildest and manifests after 20 years of age [12].

The gene for SMA, survival motor neuron 1 (*SMN1*), was cloned in 1995. *SMN1* encodes the SMN protein, which plays a critical role in cellular metabolism [22]. More than 95% of SMA patients have homozygous *SMN1* deletions [13], while the rest (< 5%) may carry intragenic *SMN1* mutations or be non-*SMN1*-related SMA (non-5q-SMA) [13]. Thus, at the beginning of diagnosis, *SMN1* deletion tests should be performed.

All SMA patients with homozygous *SMN1* deletions carry at least one copy of the *SMN2* gene, as complete loss of both *SMN* genes causes embryonic lethality [9, 27]. *SMN2* is highly homologous to *SMN1*, with only five nucleotide differences between them [11], and *SMN2* also produces a small amount of SMN protein [14]. The presence of *SMN2* may compensate for loss of *SMN1* to some degree, and higher copy numbers of *SMN2* are correlated with milder SMA phenotypes [3,7,15,29,31]. However, with regard to diagnosis or screening by conventional PCR methods, *SMN2* hampers detection of homozygous *SMN1* deletion.

To address this problem, various unique PCR methods have been established, for example, PCR and single-strand conformation polymorphism (PCR-SSCP) [13], PCR and restriction fragment length polymorphism (PCR-RFLP) [30], radio-isotope competitive PCR [15], PCR and denaturing high-performance liquid chromatography (DHPLC) [28], real-time PCR [4], multiplex ligation probe amplification (MLPA) [1], tetra-primer PCR [2],and high-resolution melting analysis[18]. However, not one of these methods can be readily introduced into all laboratories, some require expensive equipment or special reagents (e.g. radioisotopes), while others require much effort and are time-consuming procedures. Thus, further research is needed for the innovation of new simpler methods for distinguishing between *SMN1* and *SMN2*. We previously found that competitive oligonucleotide priming-PCR (COP-PCR) can separately amplify *SMN1* and *SMN2* [21], with each of the genes specifically amplified as usual by PCR.

In addition, when considering molecular analysis of any disease (including SMA), it should be noted that the capability to perform molecular analyses are not universally available in all geographical regions of the world. If simple method of collection, storage, and transfer of samples can be established, all patients will gain access to the benefits of molecular analysis regardless of geographical or industrial limitations [8]. We have previously reported that dried blood spots (DBS) on filter paper, which can be sent by mail, are a good DNA source for SMA diagnosis [8].

In this study, we combined DNA extraction from DBS on filter paper and COP-PCR analysis to establish a simple but accurate *SMN1*-deletion detection system that has practical utility in any area of the world. Here, we report on the yield and purity of DNA extracted from DBS, storage year effect on PCR amplification, and specificity of COP-PCR amplification.

MATERIAL AND METHODS

Patient and control samples

Fifty individuals were assayed, 28 SMA patients and 22 controls. All individuals had been previously analyzed by PCR-RFLP using DNA extracted from freshly collected blood [30]. Prior to analysis, informed consent was obtained from study participants. The study was approved by the Ethical Committee of Kobe University Graduate School of Medicine.

DNA extraction from DBS on filter paper

Blood samples were collected from SMA patients and controls using filter paper FTA® Elute Cards (Thermo Fisher Scientific, Waltham, MA, USA). Storage periods of blood samples on FTA® Elute Cards varied from 1 to 8 years. All FTA® Elute Cards were stored in the dark at room temperature (20–25°C). Genomic DNA was extracted from DBS according to our previous report [8]. Here, the protocol is briefly described. To extract DNA from DBS on cards, a hole punch was used to punch out seven 3-mm diameter circles from an 11-mm diameter DBS, equivalent to 50 µL of whole blood. The seven circles were placed into a sterile tube, washed in 500 µL distilled water, and vortexed three times. Washing water was completely removed from the circles, and 75 µL Tris-EDTA Buffer, pH 8.0 (TE buffer) added to the tube. The tube was heated at 95°C for 30 min, and then TE buffer containing DNA eluted and used as a DNA template for PCR or stored at −20°C until use. The

concentration and absorbance ratio of the DNA solution were measured at 260/280 nm (260/280 OD ratio) using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific).

COP-PCR for *SMN1/SMN2* exon 7

To separately amplify exon 7 from *SMN1* and *SMN2*, we performed COP-PCR using a thermal cycler (iCycler Thermal Cycler, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PCR mixture (total volume 30 μL) consisted of distilled water (22.4 μL), FastStart PCR Buffer (3 μL of 10 x concentrated buffer with 20 mM $MgCl_2$, Roche Applied Science, Mannheim, Germany), dNTP (0.4 μL of 10 mM dNTP mixture, Roche Applied Science), common forward primer (1 μL of R111 primer solution with concentration of 10 pmol/μL), gene-specific reverse primer (1 μL of SMN1-COP or SMN2-COP primer solution with concentration of 100 pmol/μL), FastStart Taq DNA polymerase (0.2 μL equivalent to 1 unit, Roche Applied Science), and DNA template (2 μL of sample solution equivalent to 50–500 ng DNA). Primer sequences are shown in Fig. 1. After an initial denaturing step of 94°C for 7 min, 30 cycles were performed consisting of 1-min denaturing at 94°C, 1-min annealing at 35°C, and 1-min extension at 72°C, followed by an additional 7-min extension at 72°C. An aliquot of each COP-PCR product was electrophoresed in a 4% agarose gel with 1×TBE buffer, and visualized by ethidium bromide staining.

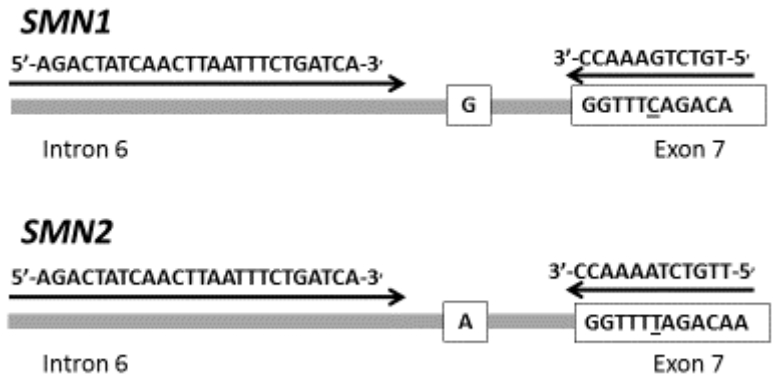

Fig. 1. Sequences, positions and directions of primers (R111, SMN1-COP and SMN2-COP). Nucleotides G and A in intron 6, and C and T in exon 7 indicate gene-specific differences between *SMN1* and *SMN2*. The expected sizes of *SMN1*- and *SMN2*-specific products are 168 bp and 169 bp, respectively.

Sequencing of COP-PCR products

Amplified COP-PCR products of *SMN1/SMN2* exon 7 were purified and used for cycle sequencing reactions with R111 primer. Cycle sequencing products were directly sequenced using a dye terminator cycle-sequencing kit (Life Technologies Corporation, Carlsbad, CA, USA). Reaction products were electrophoresed on an ABI PRISM® 310 Genetic Analyzer (Life Technologies Corporation).

RESULTS

Extracted DNA yield and storage period of DBS on filter paper

The DNA yield in an 11-mm diameter DBS circle was 21,171 ± 7,485 ng (mean ± SD), with 260/280 OD ratio from 1.49 to 2.1 (mean ± SD; 1.67 ± 0.13) (Table I).

Table I. DNA yield and 260/280 OD ratio

	DNA samples with good amplification (n=46)	DNA samples with no amplification (n=4)	All DNA samples (n=50)
DNA yield (ng) (11-mmØDBS circle)	21,564± 7,268	16,650 ± 8,403	21,171 ± 7,485
260/280 OD ratio	1.68 ± 0.13	1.60 ± 0.06	1.67 ± 0.13

There were four DNA samples (three controls and one SMA patient) with no COP-PCR amplification of either gene. Non-amplification of both genes is considered a false result, as the combination of *SMN1* and *SMN2* deletion causes embryonic lethality [9,27]. To determine if these four samples were of sufficient quality for PCR, we performed a conventional PCR experiment using a primer set for the *CFTR* gene [15], which resulted in

failure of *CFTR* fragment amplification (data not shown). Thus, we concluded that these four samples had deteriorated and were not suitable for PCR amplification, and therefore omitted them from further analysis.

These four DNA samples had been stored more than 4 years before analysis (Table II). Nevertheless, it should be noted that the DNA concentrations and 260/280 OD ratios of these samples were similar to those showing good amplification with SMN1-COP or SMN2-COP primers (Table I).

Table II. Storage period of DBS on filter paper

Years	0 - 1	2	3	4	5	6	7	8
Samples with amplification	8	6	7	4	9	7	2	3
Samples with non-amplification	0	0	0	1	0	0	1	2

COP-PCR amplification patterns

COP-PCR allowed us to separate *SMN1* exon 7 from *SMN2* exon 7 (Fig. 2). The COP-PCR diagnostic screening results are summarized in Fig. 3.

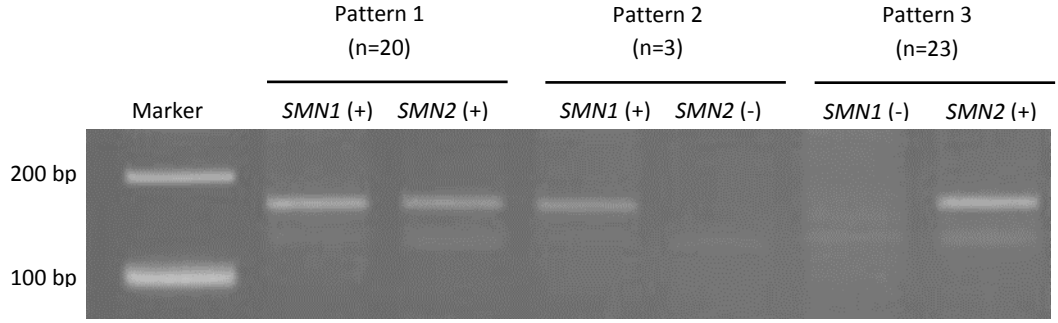

Fig. 2. COP-PCR amplification patterns. COP-PCR clearly distinguishes three genotypes: *SMN1*(+)/*SMN2*(+) (Pattern 1; *SMN1* and *SMN2* retention), *SMN1* (+)/*SMN2* (−) (Pattern 2; *SMN1* retention and *SMN2* deletion), and *SMN1*(−)/*SMN2* (+) (Pattern 3; *SMN1* deletion and *SMN2* retention).

In 18 controls and two patients with intragenic *SMN1*mutations, COP-PCR using either R111 and SMN1-COP or R111 and SMN2-COP produced amplification products (Pattern 1 in Figs. 2 and 3). In three controls, COP-PCR using R111 and SMN1-COP produced an amplification product, while R111 and SMN2-COP produced no amplification product (Pattern 2 in Figs. 2 and 3). In 23 patients with homozygous *SMN1* deletions, COP-PCR using R111 and SMN1-COP produced no amplification product, while R111 and SMN2-COP produced an amplification product (Pattern 3 in Figs. 2 and 3).

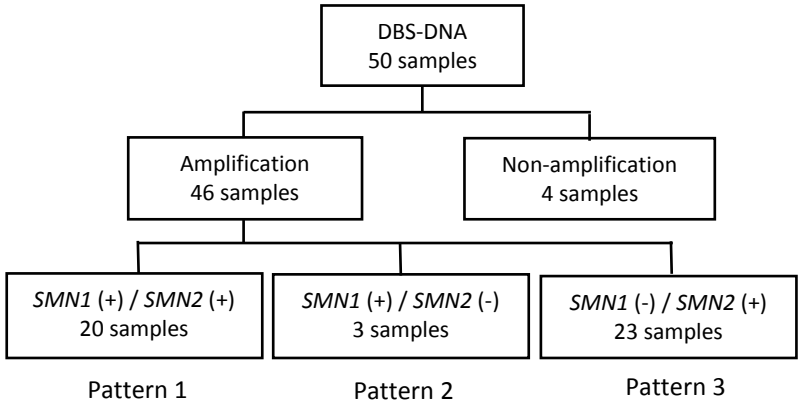

Fig. 3. Summary of the COP-PCR data. Pattern 1 group: 18 control individuals and 2 SMA patients with intragenic *SMN1* mutations. Pattern 2 group: 3 control individuals. Pattern 3 group: 23 SMA patients with homozygous deletions.

To confirm gene-specific amplification by COP-PCR, we performed nucleotide sequence analysis of COP-PCR products. According to a previous report [17], both *SMN1* and *SMN2* have gene-specific nucleotides in intron 6, with G and A nucleotides at position -44 from exon 7 of *SMN1* and *SMN2*, respectively. As shown in Fig. 4, the COP-PCR product using R111 and SMN1-COP had a G nucleotide at position -44 from exon 7, while

R111 and SMN2-COP had an A nucleotide. These findings show that COP-PCR can separately amplify *SMN1* and *SMN2* exon 7 in a gene-specific way.

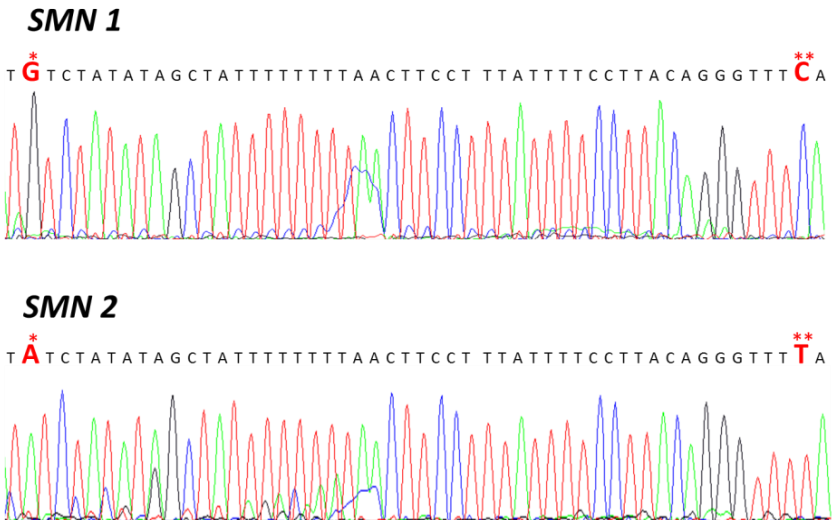

Fig. 4. COP-PCR specificity confirmed by sequencing. Nucleotide sequencing analysis confirmed *SMN1*- and *SMN2*-specific amplification. Nucleotide differences in intron 6 (*) and exon 7 (**) prove differential amplification of *SMN1* and *SMN2* by COP-PCR.

COP-PCR with DBS-DNA vs. PCR-RFLP with DNA from freshly collected blood

COP-PCR results using DBS-DNA completely matched those of PCR-RFLP using DNA from freshly collected blood (Table III). COP-PCR results using R111 and SMN1-COP also completely matched those of PCR-RFLP (Table III A). Sensitivity and specificity of COP-PCR using DBS-DNA for *SMN1* and *SMN2* exon 7 were 100%. These results suggested that COP-PCR using DBS-DNA is useful for diagnostic screening of SMA patients.

Table III. Comparison between COP-PCR and PCR-RFLP

(A)

		PCR-RFLP (freshly collected blood)		Total
		SMN1 Exon 7 Deletion	*SMN1* Exon 7 Non-deletion	
COP-PCR (dried blood)	*SMN1* Exon 7 Deletion	23	0	23
	SMN1 Exon 7 Non-deletion	0	23	23
Total		23	23	46

sensitivity 1.0, specificity 1.0

(B)

		PCR-RFLP (freshly collected blood)		Total
		SMN2 Exon 7 Deletion	*SMN2* Exon 7 Non-deletion	
COP-PCR (dried blood)	*SMN2* Exon 7 Deletion	3	0	3
	SMN2 Exon 7 Non-deletion	0	43	43
Total		3	43	46

sensitivity 1.0, specificity 1.0

DISCUSSION

DBS on filter paper

In this study, we combined DNA extraction from DBS on filter paper with COP-PCR analysis of *SMN1* and *SMN2*, establishing a simple but accurate *SMN1*-deletion detection system that has practical worldwide utility.

We used DBS-DNA as the PCR template, and obtained sufficient DNA for diagnosis of SMA. DBS on filter paper are practical and convenient with regard to handling (involves only spotting and drying blood on filter paper), transport (can be transferred by mail), storage (can be maintained at room temperature, and it is not necessary to store in a refrigerator), and cost (filter paper can be bought at minimal cost).

DBS have been used as biological specimens for more than 50 years, since Guthrie and Susi developed the phenylketonuria screening method to measure phenylalanine levels from DBS on filter paper [6]. Many technologies using DBS have already been developed and applied for medical purposes, including measurement of metabolites (e.g. adrenoleukodystrophy [20]), hormones (e.g. thyroid-stimulating hormone [11]), enzyme activities (e.g. glucose-6-phosphate dehydrogenase [26]), and drugs (e.g. antimalarial drugs [23]).

DBS are now recognized as a good source of DNA, and the combination of PCR with DNA from DBS enables genetic analysis [10, 16]. Molecular screening using DNA from DBS has already been reported for Duchenne/Becker muscular dystrophy and cystic fibrosis [25]. Our findings also support the use of DBS on filter paper as a good source of DNA for disease diagnosis. However, there are limitations to using DBS samples, and in particular, long-term storage of DBS cards may be associated with no PCR amplification, as in our study.

COP-PCR

We show here that COP-PCR can separately amplify the *SMN* genes, *SMN1* and *SMN2*. COP-PCR is a type of allele-specific amplification, in which two oligonucleotide primers compete for DNA annealing. Competitive primers are shorter than usual PCR primers (which are typically18–25 mer) and identical except for a nucleotide change that is located in the middle of the primer [5]. Amplification with the better-matched primer is favored 100-fold over the mismatched primer [5, 32]. Nucleotide sequencing confirmed that COP-PCR specifically amplified *SMN1* and *SMN2* exon 7. Comparison between COP-PCR and PCR-RFLP demonstrated a complete compatibility of both methods, proving the accuracy of COP-PCR. In addition, *SMN1*-deletion detection by COP-PCR is much faster than that by PCR-RFLP, because COP-PCR has no enzyme-digestion step. As to the required time from the start of PCR to the end of gel electrophoresis separating gene-specific products, COP-PCR takes only 3 hours, while PCR-RFLP takes more than 8 hours.

Conclusion

Here, we have combined DNA extraction from DBS on filter paper with COP-PCR that specifically detects *SMN1* and *SMN2*, and established a new *SMN1*-deletion detection system that is practical for use even in remote areas of the world. Moreover, we also demonstrate that DBS on filter paper are a good source of DNA for disease diagnosis.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

AUTHORS' CONTRIBUTION

NOZOMU KATO and NIHAYATUS SA'ADAH contributed equally to this work.

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