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Newborn Screening for Spinal Muscular Atrophy: DNA Preparation from Dried Blood Spot and DNA Polymerase Selection in PCR

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[Background] Polymerase chain reaction (PCR) analysis using DNA from dried blood spot (DBS) samples on filter paper is a critical technique for spinal muscular atrophy (SMA) newborn screening. However, DNA extraction from DBS is time-consuming, and elimination of PCR inhibitors from DBS is almost impossible. [Methods] Exon 7 of the two homologous SMA-related genes, *survival motor neuron (SMN) 1* and *SMN2*, of five SMA patients and five controls were amplified by PCR with a punched-out circle of the DBS paper. Two types of DNA preparation methods were tested; DNA-extraction (extracted DNA was added in a PCR tube) and non-DNA-extraction (a punched-out DBS circle was placed in a PCR tube). As for the DNA polymerases, two different enzymes were compared; TaKaRa Ex Taq™ and KOD FX Neo™. To test the diagnostic quality of PCR products, RFLP (Restriction fragment length polymorphism) analysis with *DraI* digestion was performed, differentiating *SMN1* and *SMN2*. [Results] In PCR using extracted DNA, sufficient amplification was achieved with TaKaRa Ex Taq™ and KOD FX Neo™, and there was no significant difference in amplification efficiency between them. In direct PCR with a punched-out DBS circle, sufficient amplification was achieved when KOD FX Neo™ polymerase was used, while there was no amplification with TaKaRa Ex Taq™. RFLP analysis of the direct PCR products with KOD FX Neo™ clearly separated *SMN1* and *SMN2* sequences and proved the presence of both of *SMN1* and *SMN2* in controls, and only *SMN2* in SMA patients, suggesting that the direct PCR products with KOD FX Neo™ were of sufficient diagnostic quality for SMA testing. [Conclusion] Direct PCR with DNA polymerases like KOD FX Neo™ has potential to be widely used in SMA newborn screening in the near future as it obviates the DNA extraction process from DBS and can precisely amplify the target sequences in spite of the presence of PCR inhibitors.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive disease exhibiting muscle atrophy and weakness of the whole body due to degeneration or loss of motor neurons in the anterior horn of the spinal cord. In 1995, SMA-related genes, *survival motor neuron* (*SMN*) *1* and *SMN2* genes, were identified and successfully cloned[7]. *SMN1* and *SMN2* are highly homologous genes, but succeeding studies clarified that *SMN1* is responsible for

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SMA[13]. According to our previous study[9], the incidence rate of the infantile onset SMA is ~1/20,000 newborn babies, and its carrier rate is ~1/70 in Japan.

SMA cannot be definitely diagnosed by clinical symptoms alone and it should be confirmed by detection of *SMN1* abnormalities[1, 13]. Advantages of such genetic test are minimal invasiveness and high accuracy for the disease diagnosis. Nevertheless, the availability of the genetic test has not been well recognized, resulting in delayed or missed diagnosis in some cases[9]. In addition, SMA was considered as a disease without treatment options, which might be related to the delayed diagnosis of SMA.

However, much progress has been made in therapeutic strategies for SMA. An antisense oligonucleotide drug for SMA, nusinersen, has recently been developed, and its clinical trials have shown promising clinical effects on the patients[3,4]. In 2016, nusinersen was approved for clinical use in the US, and in 2017, it was also approved in Japan. The clinical trial results also indicated that early start of treatment can produce more effective efficacy and thus delayed diagnosis would impair the potential of treating patients early.

Since early treatment is related to early diagnosis and recognition of the disease in patients, the feasibility of implementing newborn screening for SMA should be discussed[9]. Dried blood spot (DBS) on the filter paper is used for nationwide newborn screening to detect inborn errors of metabolism in Japan. Thus, inclusion of genetic test for SMA using DBS would enable us to carry out the nationwide newborn screening for this disorder on the currently available screening platform. Nationwide newborn screening for SMA can decrease the rate of delayed diagnosis of SMA in Japan.

For SMA newborn screening with genetic testing, polymerase chain reaction (PCR) using DBS is an essential technique. Previous studies, including ours, have shown successful DNA extraction from DBS paper[5,8]. However, the DNA extraction process was time consuming. In addition, DBS paper contains various PCR inhibitors by which some DNA polymerase does not work as expected[12]. Thus, we investigated the method of the optimal DNA preparation and DNA polymerase selection from DBS samples. In the current study, we compared the representative enzymes of two distinctive DNA polymerase groups. TaKa Ra Ex Taq™ is an advanced version of the most popular thermophilic DNA polymerase, Taq, which was isolated from Thermus aquaticus[10]. KOD FX Neo™ is also an advanced version of KOD DNA polymerase, which was isolated from Thermococcus kodacaraensis[10]. KOD has 3'→5' exonuclease activity, so the fidelity of KOD polymerase is much better than Taq[10].

MATERIALS AND METHODS

1. DNA preparation from DBS

The participants in this study were five SMA patients and five healthy controls (volunteers). The patients had been diagnosed by genetic test using freshly collected blood. Prior to this study, informed consent was obtained from all participants who provided their whole blood for the study. This study was approved by the Ethics Committee of Kobe University Graduate School of Medicine (No. 1089).

DBS samples were prepared as follows: EDTA-anticoagulated fresh blood was spotted on the filter paper (Toyo Roshi; Advantec MFS Inc. Tokyo, Japan) and allowed to air dry for more than 2 hours. DNA was prepared from DBS according to the 5 different methods of (A)-(E). (A), (B), (C) and (D) were grouped into the "DNA extraction" method, while (E) was grouped into the "non-DNA-extraction" method. The details of each method is as follows:

(A) DNA extraction by a simple "boiling" method: a punched-out DBS circle with a 2mm-diameter (DBS disk) was placed in 50 μL of TE buffer and boiled at 95°C for 30 minutes. DNA was eluted in the solution. Two μL of the DNA solution was then added into PCR reaction mixture.

(B) DNA extraction by a "rinsing and boiling" method: a DBS disk was rinsed with Milli-Q (MQ) water, and then placed in 50 μL of TE buffer and boiled at 95°C for 30 minutes. Two μL of the DNA solution was then added into PCR reaction mixture.

(C) DNA extraction by a "protein fixation-1, rinsing and boiling" method: a DBS disk was placed in 100 μL of a mixed solution containing acetone, methanol, and water (7:7:2, volume ratio) (protein fixation)[8]. After protein fixation, the disk was rinsed with MQ water, and then placed in 50 μL of TE buffer and boiled at 95°C for 30 minutes. Two μL of the DNA solution was then added into PCR reaction mixture.

(D) DNA extraction by a "protein fixation-2, rinsing and boiling" method: a DBS disk was placed in 100 μL of methanol (protein fixation). After protein fixation, the disk was rinsed with MQ water, and then placed in 50 μL of TE buffer and boiled at 95°C for 30 minutes. Two μL of the DNA solution was then added into PCR reaction mixture.

(E) No DNA extraction process: A half of the DBS disk was directly placed into a PCR tube, that is, PCR was performed without DNA extraction.

2. PCR experiments with two different DNA polymerases

All PCR experiments were carried out using a Thermal Cycler GeneAtlas (Aztec Inc. Tokyo, Japan). DNA extracted from DNA solution of (A), (B), (C) and (D) (2μL each), and one half of the DBS disk (E) was used as template for PCR. Two μL of DNA solution of (A), (B), (C) and (D) contained ~100 ng DNA.

Two DNA polymerases, TaKaRa Ex Taq™ (Takara Bio Inc. Shiga, Japan) or KOD FX Neo™ (Toyobo Inc. Osaka, Japan) were used in this study. Each PCR experiment was performed with 50 μL of the reaction mixture according to the instruction manuals of the companies. PCR primer set of R111 (forward primer) [7] and X7-Dra (reverse primer) [11] was used in this study. The PCR conditions were as follows; 1). Initial denaturation: 94°C for 7 minutes; 2). PCR cycles: 94°C for 1 minute, 56°C for 1 minute and 72°C for 1 minute. The cycle numbers were 25, 30, 35, or 40 cycles; and 3). Final extension: 72°C for 7 minutes. The PCR products were electrophoresed on a 4% agarose gel and visualized with ethidium bromide.

3. PCR-RFLP analysis with *DraI* digestion

To test the diagnostic quality of the PCR products, PCR-RFLP (Restriction fragment length polymorphism) with *DraI* digestion was performed differentiating *SMN1* and *SMN2*. *DraI* site was introduced into *SMN2* product during the PCR[11]. The PCR product was digested by overnight incubation with *DraI*. More specifically, 8 μl of the PCR products was added to the enzyme solution (final volume, 20 μl) containing 1 × buffer M [final concentration] and 1 μl of *DraI* (15 U/μl) (Takara Biomedicals, Shiga), and the mixture was incubated overnight at 37°C. Subsequently, an aliquot of digested product was electrophoresed on a 4% agarose gel, and visualized with ethidium bromide.

After *DraI* digestion, the *SMN2* product (187 bp) generated two fragments of 163 bp and 24 bp, while the *SMN1* product (187 bp) did not undergo *DraI* digestion and retained the same product size as the non-digested fragment.

RESULTS

1. Comparison between DNA preparations and between DNA polymerases

The experiments for the comparison between DNA preparations and between DNA polymerases were done using a control sample.

When TaKaRa Ex Taq™ polymerase was used, PCR with DNA preparation methods (A), (B), (C) and (D) generated high amount of PCR products at 40 cycles and the agarose gel electrophoresis showed clear bands of the PCR products. However, PCR with DNA preparation method (E) did not generate enough amount of PCR products at 40 cycles and the agarose gel electrophoresis showed no band of the PCR product (Fig.1).

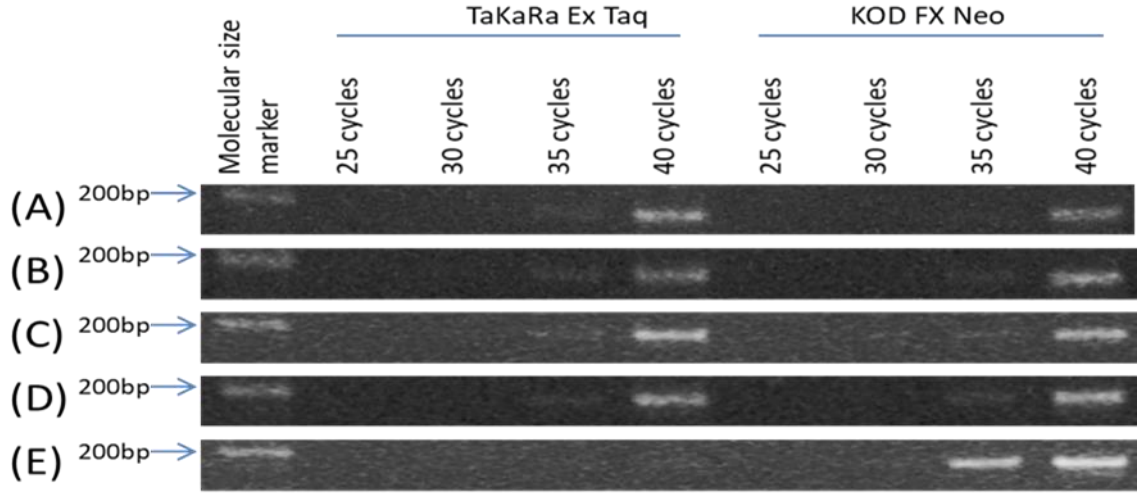

Fig. 1. Comparison between DNA preparations and between DNA polymerases

DNA preparation methods were as follows: (A) DNA extraction by a simple "boiling" method, (B) DNA extraction by a "rinsing and boiling" method, (C) DNA extraction by a "protein fixation-1, rinsing and boiling" method, (D) DNA extraction by a "protein fixation-2, rinsing and boiling" method, and (E) No DNA extraction process. (A), (B), (C) and (D) were grouped into the "DNA extraction" method, while (E) was grouped into the "non-DNA-extraction" method. DNA polymerases tested in this study were TaKaRa Ex Taq™ and KOD FX Neo™.

When KOD FX Neo™ polymerase was used, PCR with DNA preparation methods (A) to (D) generated high amount of PCR products at 40 cycles. It should be noted that PCR with DNA preparation method (E) using KOD FX Neo™ also generated high amount of PCR products at 35 and 40 cycles.

The difference of PCR amplification efficiency with KOD FX Neo™ between (E) and others (A-D) may be due to the abundance of DNA. DNA in samples (E) was much more abundant than DNA in samples (A-D); one half of the DBS disk (E), which was used as template for PCR, was much more abundant in DNA than 2 μL of DNA solution of (A), (B), (C) and (D).

2. Test of the diagnostic quality for SMA

To test whether a combination of the method (E) and KOF FX Neo™ polymerase (i.e., direct PCR with KOF FX Neo™ polymerase) can produce sufficient and qualified PCR products for SMA diagnosis, we performed PCR-RFLP analysis with *DraI* digestion.

The agarose gel electrophoresis of the PCR products obtained at 40 cycles showed clear bands of *SMN1* and/or *SMN2* (Fig. 2, upper panel). The results of PCR-RFLP showed two bands of the PCR products from *SMN1* and *SMN2* in the five healthy volunteers, whereas only one band of the PCR products from *SMN2* in five SMA patients (Fig. 2, lower panel). All SMA patients analyzed here had complete *SMN1* deletion. These results were perfectly consistent with those of PCR-RFLP analysis using freshly collected blood samples (data not shown).

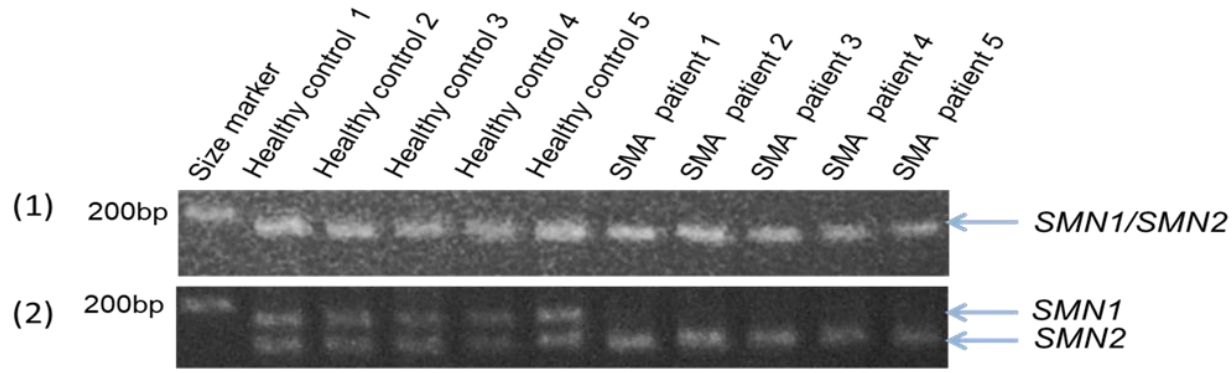

Fig.2. RFLP using direct PCR products with KOD FX Neo™ polymerase

(1) Before *DraI* digestion. The bands represent the mixture of PCR products from *SMN1* and/or *SMN2*. (2) After *DraI* digestion. Upper bands represent PCR products from *SMN1* and lower bands PCR products from *SMN2*. *DraI* digestion proved the presence of *SMN1* and *SMN2* in the PCR products of the healthy volunteers and the absence of *SMN1* and the presence of *SMN2* in the SMA patients. All SMA patients showed the absence of *SMN1*.

DISCUSSION

Here, we investigated whether PCR can be performed with or without DNA extraction process from DBS. When TaKaRa Ex Taq™ polymerase was used, DNA extraction process might be essential. Without DNA extraction process, PCR amplification was not successful even at 40 cycles. Whereas, when KOD FX Neo™ was used, PCR amplification was successful without DNA extraction process; PCR products at both 35 and 40 cycles were clearly detected on the gel electrophoresis (Fig. 1). These results indicate that KOD FX Neo™ is much less affected by PCR inhibitors presented in the DBS paper, and that we are able to perform direct PCR from the DBS paper without DNA extraction process.

We have known from our experience that KOD FX Neo™ polymerase is useful for long-range PCR targeting at amplification of more than 25Kb sized DNA such as *SMN* genes[6]. However, we used it in this study, not for long-range PCR (the PCR product sizes in this study was less than 200bp), but for direct PCR with a piece of DBS paper. According to the information from the company website (http://lifescience.toyobo.co.jp/), "the KOD FX Neo™ is able to amplify DNA from crude samples and can reduce influence of PCR inhibitors within soil, food, etc." Our results clearly proved that KOD FX Neo™ polymerase is useful for the PCR amplification from DBS which can be considered as a kind of crude sample. Although the company emphasizes that KOD FX Neo™ overcomes PCR inhibitors in the crude samples, it may be empirical data, because we failed to get clear explanation about the mechanism of KOD FX Neo™ in the previous literatures.

If the diagnostic quality of direct PCR products is guaranteed, DNA extraction process from DBS can be omitted from the DBS-based SMA screening system. Bypassing the DNA extraction process can simplify the DBS-based SMA screening system and also reduce the required time for the screening. Thus, we performed

RFLP analysis to test the diagnostic quality of the direct PCR products with KOD FX Neo™ polymerase. The results of RFLP analysis showed that *SMN1* and *SMN2* were present in all of five healthy volunteers, whereas only *SMN2* in all of the five SMA patients. These findings were completely compatible with those of PCR-RFLP analysis with freshly collected blood samples, indicating that the diagnostic quality of direct PCR products was guaranteed.

In conclusion, direct PCR with DNA polymerases like KOD FX Neo™ can be widely used in newborn screening for SMA in the near future, because it can skip the process of DNA extraction from DBS and precisely amplify the target sequences in spite of the presence of PCR inhibitors. We previously established a DBS-based SMA screening with a modified competitive PCR (mCOP-PCR) technique[2]. In the previous protocol, a DNA extraction process was needed. However, the results of the current study may enable us to develop more sophisticated DBS-based newborn screening system for SMA, which can significantly reduce both process time and work load.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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