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Performance of cellulose-based card for direct genetic testing of spinal muscular atrophy

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Abstract

Background Spinal muscular atrophy (SMA) is a devastating neuromuscular condition resulting from the loss of the *survival motor neuron 1 (SMN1)* gene. Precise genetic testing has become essential after the authorization of several potent medications. To achieve this objective, the use of dried blood spot (DBS) has assured convenient and extensive testing from a distance. Nevertheless, developing countries such as Indonesia sometimes lack access to standard filter papers like FTA or Guthrie cards for DBS processing. Here, we aim to develop a cellulose-based card as an alternative filter paper for DBS preparation suitable for the genetic testing of SMA including but not limited to a direct polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and multiplex allele-specific amplification (multi-ASA).

Results An in-house paper was developed from a 180 gsm cellulose-based paper and was used for DBS preparation. The performance of dried blood spotted on the cellulose-based card (DBSc) was compared to pure genomic DNA (gDNA) isolate and dried blood spotted on FTA cards (DBSf) for genetic testing. The results of the genetic testing of our cellulose-based card were completely matched with those of gDNA and DBSf in both direct PCR-RFLP and Multi-ASA to separate *SMN1* from *SMN2*. In addition, after three months of storing, the DBSc continued to exhibit a clear result, suggesting its high stability for DNA storage.

Conclusion Our cellulose-based card has the potential to be used for DBS carrier and for further genetic testing using PCR. Our findings can assist physicians in sending DBS samples from SMA suspicion cases to genetic testing centers, thereby preventing diagnosis delay or misdiagnosis.

Keywords Filter paper, Spinal muscular atrophy, *SMN1*, *SMN2*, Dried blood spot, Direct PCR

Introduction

Spinal muscular atrophy (SMA), characterized by motor neuron degeneration, muscle weakness, and muscular atrophy, is one of the most devastating neuromuscular disorders [1]. It is an autosomal recessive disease that is passed down from parent to child. Most individuals with this condition exhibit either a loss or a mutation within the *survival motor neuron 1* (*SMN1*) gene [1, 2]. All SMA patients retain the homologous gene, *survival motor neuron 2* (*SMN2*), which possesses similar sequences except for five different locations, with the exon 7 location playing an important role in SMA pathogenesis [1]. As a

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result, SMA molecular diagnosis has focused on detecting *SMN1* exon 7. Several methodologies have been developed for genetic testing of SMA including single-stranded conformation polymorphism (SSCP) analysis [1], restriction enzyme digestion analysis [3], modified competitive oligonucleotide priming-polymerase chain reaction (mCOP-PCR) [4, 5], allele-specific amplification [6, 7], and multiplex ligation-dependent probe amplification (MLPA) [8].

Genetic testing for SMA has become increasingly important following the approval of new drugs which improve patient prognosis if administered early before symptoms appear [9–11]. Consequently, many countries have even incorporated SMA screening into their nationwide newborn screening policy, which utilizes dried blood spots (DBS) on filter paper as the starting material [4, 12–19].

Despite the importance of SMA genetic testing, developing countries such as Indonesia face many challenges for SMA genetic testing due to lack of resources, not to mention the complexity of cold-chain sample transportation to genetic testing centers from remote areas. The use of DBS on a standard filter paper such as Guthrie cards or FTA cards is a convenient method to address this challenge due to its simple preparation, transportation, storage, and analysis [20–23]. It even enables researchers to diagnose SMA across different countries [24]. However, these standard cards may not always be widely available in certain countries where resources are limited and import regulation is strict. These standard cards are not only expensive but also hard to find in Indonesia.

In this study, we aimed to develop an in-house, cellulose-based card as an alternative card suitable for DBS carrier and downstream molecular analysis for SMA detection including but not limited to polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism (RFLP) and multiplex allele-specific amplification PCR (Multi-ASA-PCR). Our results will help clinicians diagnose SMA suspects from remote areas across the country.

Methods

Ethics approval and consent to participate

Informed consent was obtained from all probands prior to this study. This study was approved by the Medical and Health Research Ethics Committee (MHREC) at the Faculty of Medicine, Nursing, and Public Health, Universitas Gadjah Mada (reference number KE-FK-0467-EC-2023, approved on March 2023) and was conducted following the World Medical Association Declaration of Helsinki.

Dried blood samples

The cellulose-based card was made from 180 gsm cellulose paper. We treated the filter paper by autoclaving it at 121 °C and 20 psi for 20 min and air-drying it before usage. Standard filter papers, Flinders Technology Associated (FTA) cards, were purchased from Genetika Science Indonesia (Jakarta, Indonesia) and were used as control standard cards.

Dried blood spots were made by spotting 50 µL of whole blood from 20 SMA patients and 42 healthy probands onto the cellulose-based card and FTA card, which were then left to air dry at room temperature for one hour. We then added 3–5 drops of absolute methanol to the DBS spotted onto the cellulose-based card but not FTA card. Both the dried blood spotted onto the cellulose-based card (DBSc) and dried blood spotted onto the FTA card (DBSf) were stored at room temperature in a dark room with desiccants until analysis.

Genomic deoxyribonucleic acid (gDNA) was extracted from the remaining whole blood of the probands using a FavorPrep™ Blood/Cultured Cell Genomic DNA Extraction Mini Kit (Favorgen, Ping Tung, Taiwan) or a Sepagene Kit (Sanko Junyaku, Tokyo, Japan) according to the manufacturer's protocol. The concentration and purity of the products were determined using a NanoDrop spectrophotometer (MaestroNano, Hsinchu, Taiwan). The gDNA was used as the standard starting material for PCR comparison.

Molecular analysis

We evaluated the performance of DBSc and DBSf for direct conventional PCR-RFLP skipping DNA isolation step. For every PCR analysis, a single 1.2 mm diameter punch from both DBSc and DBSf was used as the PCR template. Approximately 50–100 ng of gDNA was also used as the gold standard template. All PCRs were performed using KOD FX Neo™ (Toyobo, Osaka, Japan) in a final volume of 50 µL. This included 1X PCR buffer, 0.4 mM of each dNTP, 0.3 µM of each primer, 1.0 U of KOD FX NEO, one punched of DBS (DBSc or DBSf) or 50–100 ng of gDNA. The sequences and estimated fragment size of all the primers used in this study are given in Supplementary Table 1. Either a T100 Thermal Cycler (Bio-Rad) or a Veriti™ 96-well Fast Thermal Cycler (Applied Biosystems/Thermo Fisher Scientific) was used for thermal cycling.

PCR-RFLP

We evaluated the performance of DBSc and DBSf for PCR-RFLP. *SMN exon 7* and *SMN exon 8* were first PCR amplified using the common *SMN exon 7* and *SMN exon 8*, respectively (Supplementary Table 1). The PCR conditions for both *SMN exon 7* and *8* were as follows: one cycle of initial denaturation at 98 °C for 2 min, 40 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s. The procedure concluded with a final elongation at 72 °C for 7 min. Five

microliters of the PCR products were subjected to 3% agarose gel electrophoresis. A total of 15 µL of the *SMN exon 7* PCR products was subjected to enzyme digestion containing the DraI enzyme (New England Biolabs) and 1X buffer in a final volume of 20 µL and incubated at 37 °C overnight. A total of 10 µL of the *SMN exon 8* PCR products was subjected to enzyme digestion containing the DdeI enzyme (New England Biolabs) in a final volume of 12 µL and incubated at 37 °C overnight. The digested products were then electrophoresed on a 4% agarose gel.

Multi-ASA-PCR

Multiplex allele-specific amplification-PCR was evaluated by amplifying *NAIP*, *SMN1* exon 7 and the *CFTR* gene. Multi-ASA-PCR was performed in a 50 µL final volume containing *NAIP*, *SMN1 exon 7* and *CFTR* specific primer pairs (Supplementary Table 1) with the following conditions: one cycle of initial denaturation at 98 °C for 2 min, 40 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. This was finalized with a final elongation at 72 °C for 7 min. Five microliters of the PCR products were subjected to 3% agarose gel electrophoresis.

Results

Dried blood spotted on the cellulose-based paper successfully amplified *SMN1*

This study was approved by the Ethics Committee of Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (approval number KE-FK-0467-EC-2023). To evaluate whether our in-house, cellulose-based card was suitable for use for DBS carrier and downstream molecular analysis, we prepared DBSc and DBSf from 20 genetically known SMA patients and 42 healthy controls. Genomic DNA (gDNA) was parallelly isolated from respective probands for comparison because gDNA is the gold standard for genetic testing starting material. All DBSc, DBSf, and gDNA were subjected to *SMN1* deletion test using PCR-RFLP to separate *SMN1* from *SMN2*.

First, we performed direct PCR-RFLP in which the DNA isolation process from DBSc or DBSf was skipped. One punch, 1.2 mm in diameter, of the DBSc or DBSf was directly put into the PCR reaction mixture before running the amplification. This way, we shortened the turnaround time for genetic testing from the DBS sample. For all DBSc, DBSf, and gDNA, we maintained the same PCR condition and final reaction mixture volume.

We found that the results of direct PCR-RFLP from DBSc were completely matched with DBSf and gDNA where *SMN1 exons 7 and 8* and *SMN2 exons 7 and 8* could be separated precisely (Fig. 1A and B). In the agarose gel electrophoresis pattern, healthy DBSc samples had an undigested 187 bp of *SMN1 exon 7* fragment and a digested 163 bp of *SMN2 exon 7* fragments. However, DBSc of SMA samples only had one 163 bp of *SMN2 exon 7* fragments.

Similarly, healthy DBSc samples showed a clear *SMN1 exon 8* fragment (189 bp) and *SMN2 exon 8* fragments (digested, 123 bp, and 66 bp), whereas DBSc samples from SMA only showed *SMN2 exon 8* fragments (123 bp and 66 bp). The results of direct PCR-RFLP from the cellulose-based card were also matched with those of standard FTA cards or its respective gDNA, indicating the potential of cellulose-based card to be used as an alternative card for genetic testing of SMA.

The DBSc was applicable for multiplex-allele specific amplification-PCR (Multi-ASA-PCR)

We further tested the ability of DBSc for multi-ASA-PCR by co-amplified three gene fragments at the same time: *neuronal apoptosis inhibitory protein* (*NAIP*), *SMN1 exon 7*, and *cystic fibrosis transmembrane conductance regulator* (*CFTR*). The use of allele-specific amplification allows for the reduction of the turnaround time for genetic testing of SMA suspicion cases compared to conventional PCR-RFLP, while also obtaining a comprehensive data simultaneously. *NAIP* gene is located on the same region as the *SMN1* (chromosome 5q13) gene, and deletion of this gene is often associated with severe forms of SMA, particularly type I [25–29]. Thus, a deletion test of the *NAIP* gene can help physicians make precise diagnoses or predict prognosis. In this study, co-amplification of a housekeeping gene, *CFTR*, served as a template control to ensure the presence of a template during the reaction, especially in severe SMA patients who suffered from both *NAIP* and *SMN1* deletion.

Figure 2 shows a representative result of multi-ASA-PCR analysis of DBSc, gDNA, and DBSf from the same probands. The analysis was designed to amplify three gene fragments from *NAIP* (436 bp), *SMN1 exon 7* (307 bp), and *CFTR* (237 bp). This way, we incorporated the homozygous *SMN1* deletion test and the homozygous *NAIP* deletion test in a single multi-ASA-PCR. As seen in Fig. 2, healthy probands showed the presence of all three fragments, whereas SMA patients only showed two fragments of NAIP and CFTR. The result of multi-ASA-PCR was consistent with PCR-RFLP, with all genetically known SMA patients analyzed in this study consistently demonstrating no amplification of *SMN1* exon 7. This suggests that multi-ASA-PCR has a good accuracy to be used for genetic testing of SMA.

Additionally, we identified one healthy proband who exhibited a deletion of the *NAIP* fragment in the multi-ASA-PCR (data not presented). Although the NAIP gene deletion was associated with a severe form of SMA, this condition was not exclusive to SMA patients and can occur at a low frequency in unaffected individuals or carrier parents, indicating that NAIP deletion alone is

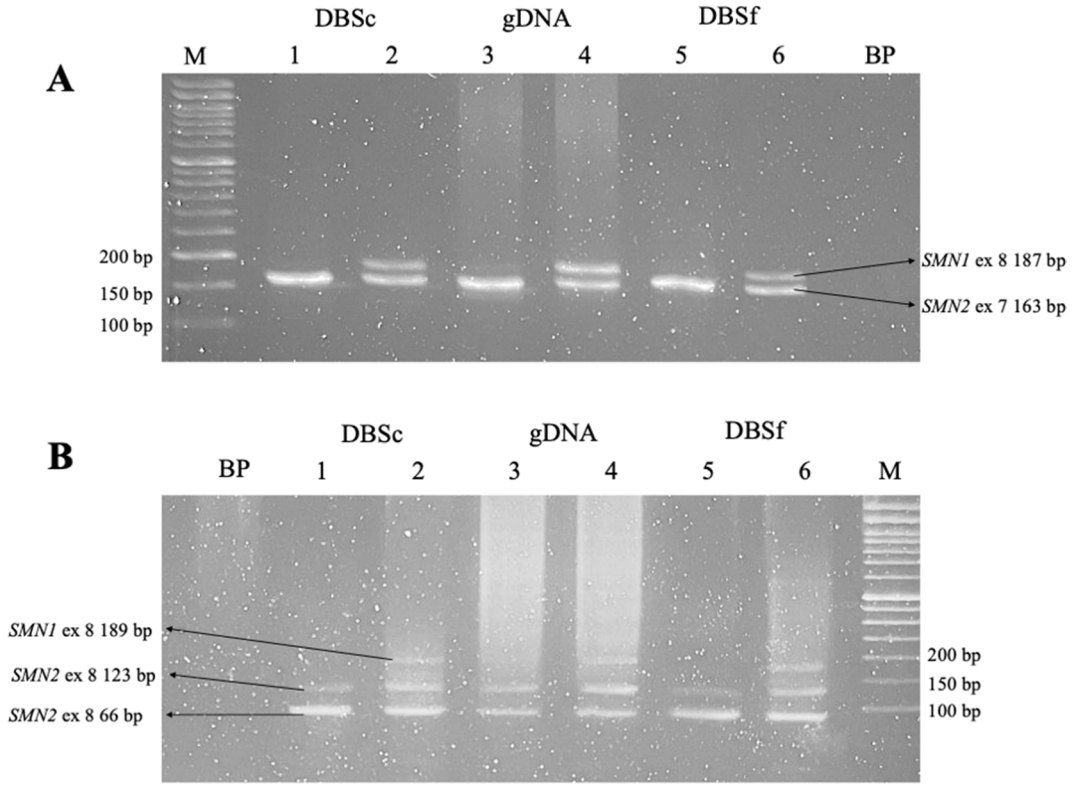

Fig. 1 PCR-RFLP results from the dried blood spots on the cellulose-based card (DBSc), FTA card (DBSf) and gDNA. **A** Electropherogram of *SMN exon 7* digestion showing *SMN1 exon 7* fragment (187 bp) and *SMN2 exon 7* fragment (163 bp). **B** Electropherogram of *SMN exon 8* digestion showing *SMN1 exon 8* fragment (189 bp) and *SMN2 exon 8* fragments (123 bp and 66 bp). M = DNA ladder with indicated fragment size. Lanes 1, 3 and 5 were from SMA patients. Lanes 2, 4, and 6 were from healthy proband. BP = blank paper

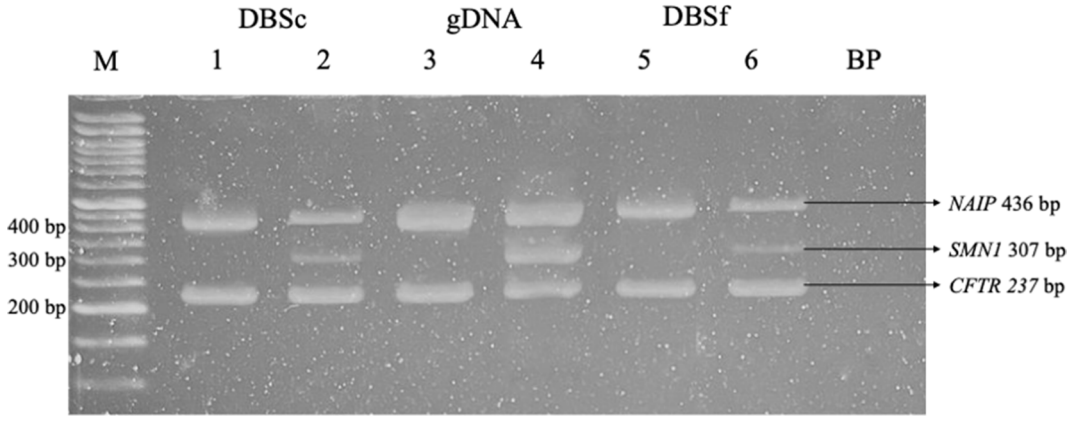

Fig. 2 Multiplex-allele-specific amplification-polymerase chain reaction (multi-ASA-PCR) from DBSc, gDNA and DBSf. Multi-ASA-PCR co-amplified *NAIP* (436 bp), *SMN1 exon 7* (307 bp), and *CFTR* (237 bp). M = DNA ladder with indicated fragment size. Lanes 1, 3 and 5 were from SMA patients. Lanes 2, 4, and 6 were from healthy proband. BP = blank paper

insufficient to cause the disease [29–31]. Nevertheless, the *NAIP* deletion test, in conjunction with the *SMN1* deletion test, was a critical component of SMA molecular testing, as it facilitated in understanding the disease's severity and guiding clinical management [26, 29, 32].

The effect of storage period of dried blood spotted on the cellulose-based card

In this study, direct PCR was performed from a relatively fresh prepared DBSc collected from probands. To determine whether DBSc could continue to function as a template for PCR-based genetic testing, we periodically made a new DBSc from the same healthy proband

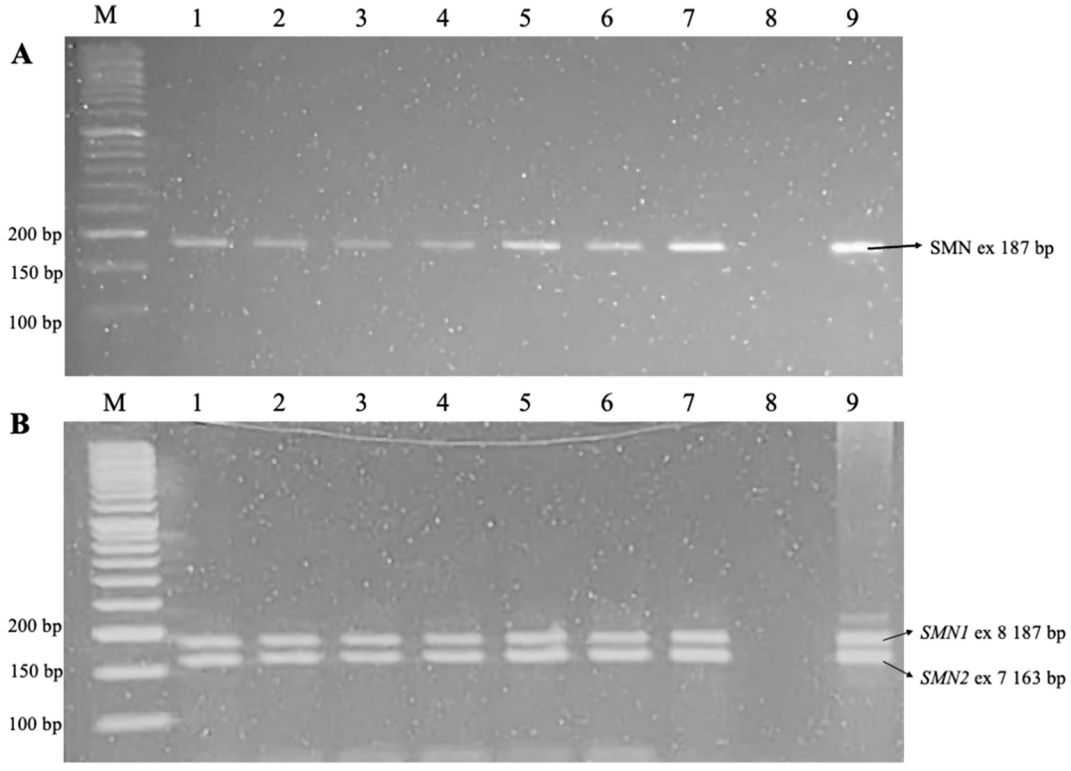

Fig. 3 PCR-RFLP of *SMN* exon 7 results of DBSc over storage period. **A** Pre-digestion PCR product of SMN exon 7 (187 bp). **B** Post-digestion PCR products showing *SMN1* exon 7 fragment (187 bp) and *SMN2* exon 7 fragment (163 bp). M = DNA ladder. Lane 1 = DBSc stored for 1 day, Lane 2 = DBSc stored for 1 week, Lane 3 = DBSc stored for 2 weeks, Lane 4 = DBSc stored for 3 weeks, Lane 5 = DBSc stored for 4 weeks, Lane 6 = DBSc stored for 2 months, Lane 7 = DBSc stored for 3 months, Lane 8 = control blank paper, Lane 9 = gDNA

and used them as a template for PCR-RFLP. We found that the results of PCR-RFLP remained consistent over time, where both *SMN1* and *SMN2* were amplified from the healthy proband even after a storage period of three months (Fig. 3). This finding indicated that the DNA stored in the cellulose-based card remained stable for PCR-based analysis for at least 3 months. We stored the DBS in a single, dry, airtight zipper bag at room temperature (around 25 °C) with silica gel desiccant to prevent humidity. The zipper bag containing the DBS was protected from UV light exposure by keeping it in a dark place. DBS storage conditions must be controlled to maintain DNA integrity for molecular analysis [33, 34]. When properly stored, dried blood spotted on standard filter paper like FTA card can be used for genetic analysis after years of storage [35]. At the time this manuscript was written, the effect of the storage period was still continuing. The duration for which the DNA can be stably stored on our cellulose-based card for genetic testing has not yet been established in this study.

Discussion

Approaches to optimize cellulose-based card as DBS carrier

Here, we tested cellulose-based card as an alternative DBS carrier and performed SMA genetic testing from it. The physical characteristics and fiber density of our card closely resembled those of standard cards, where 180 gsm paper is deemed suitable for analysis of human fluids, including blood [36]. The card was dense enough to entrap cells containing nucleic acid, which is necessary for genetic analysis.

Several factors need careful attention to ensure the success of downstream molecular analysis from a crude sample. One critical aspect is the removal of any remaining DNAse activity from the DBS. Trace amount of DNAse present in the DBSc may originate from either the card (DNAse expressed by plants) or the blood [37–40]. There are two families of DNase enzymes (DNAse I and II) that are able to hydrolyze the phosphodiester bonds of DNA molecules, leading to the degradation of DNA molecules [41]. Fragmentation of DNA in DBSc might affect downstream analysis, including amplification of large fragments. This issue could limit the potential of DBSs for genetic analysis and shorten the storage period of DBSs.

Thus, inhibiting DNAse activity is crucial for optimizing cellulose-based card potentials.

There are some approaches to maximize DNAse activity inhibition. One such approach is to employ chelator agents such as ethylene-diamine-tetraacetic acid (EDTA) or ethylene-glycol-tetraacetic acid (EGTA) during blood collection. Both agents are inexpensive reagents that inhibit DNAse activity to such an extent [38, 41]. Another method for inhibiting DNAse activity involves treating filter paper with diethylpyrocarbonate (DEPC), a potent and nonspecific inhibitor of DNAse and RNase. However, this chemical is carcinogenic and requires strict attention for biosafety [42, 43].

An easy and relatively safer method for inhibiting DNAse activity is through intense heating with an autoclave for 15 min at 121 °C [44]. Apart from irreversibly inactivating DNAse on the card, autoclaving could also inactivate DEPC and degrade DNA, suggesting that the order of method used for DNAse inactivation needs to be strictly followed [44, 45]. In this case, autoclaving should be performed after DEPC treatment and before blood spotting.

In this study, we performed relatively simple and safe approaches to maximize DNAse inhibition. First, the cellulose-based card underwent autoclave procedure before being used for making blood spots. This pre-treatment was intended to eliminate trace DNAse present in the card. In addition, we used EDTA as an anticoagulant during blood collection before spotting the blood on the cellulose-card to further maximize DNAse inhibition. EDTA was preferred over heparin as the anticoagulant for DBS preparation because it can inhibit DNAse and is less inhibitory for PCR reaction [38, 41, 46]. PCR inhibition from anticoagulant including heparin could increase the risk of false results [46].

Furthermore, we also treated the DBSc with methanol fixation by adding a few drops of methanol after spotting whole blood onto the cellulose-based card. Methanol is widely used as a DNA preservation agent [47–49]. This methanol fixation treatment works by dehydrating and denaturing proteins and damaging cellular structures but has little effect on nucleic acid degradation, enabling genetic analysis to be performed on biological samples [47–50]. The combination of autoclaving, EDTA, and methanol fixation enabled the cellulose-based card to store DNA from blood spots for an extended period of time. However, further studies need to be conducted to test how long DBSc can be used for genetic analysis.

Another essential consideration for genetic analysis using DBS to facilitate remote genotyping is the transportation conditions. Although transportation of DBS does not require complex cold-chain protocols, it is crucial to protect DBS from UV exposure and humidity during shipping. This can be achieved by simply enclosing them in a sealed envelope with silica gel. This straightforward transportation process allows reliable genetic analysis from DBS both domestically [4] and internationally [24].

Based on the discussion above, an optimized alternative DBS carrier might be useful to support remote SMA diagnosis, particularly in resource-limited settings, thus preventing diagnosis delay or misdiagnosis in highly suspicious cases. Although DBS has been found to be simple, it still can maintain good accuracy for the *SMN1* homozygous deletion detection to diagnose SMA from different countries across the globe [24, 51]. One report even suggested the feasibility to screen for SMA carriers by determining *SMN1* copy number from DBS [24]. The broad versatility of DBS for genetic testing has therefore supported a more comprehensive management for SMA suspicious cases and provided valuable information for genetic counseling and family planning, especially in resource-limited settings [24].

Limitations of cellulose-based card

It should be emphasized that while DBSc has some benefits, it has constraints in achieving precise diagnosis for all SMA cases. While our study reported a favorable accuracy rate for SMA patients with complete *SMN1* deletions, *SMN1* deletion test alone was not always sufficient since there was a few SMA patients who suffered from various types of mutation in their remaining *SMN1* gene, including missense, nonsense, splice site, and frameshift mutations [1, 52–55]. For these cases, determination of *SMN1* copy number followed by sequencing should be performed to look for any deleterious mutation, and the occurrence of mutation should be verified in *SMN1*, not *SMN2* [52, 55]. To achieve this, a long-range PCR might first be needed to specifically separate *SMN1* from *SMN2* before performing nested PCR on *SMN1 exons* for mutation hunting [52, 55, 56]. This way, clinicians can verify the exact location of the mutation, which is essential for the diagnosis of SMA.

Currently, our method could not quantify *SMN1/SMN2* copy number limiting the scope of PCR analysis that can be carried out from DBSc. Unlike emerging techniques such as MLPA which not only identified *SMN1* deletion but also quantified *SMN1/SMN2* copy numbers [8], our system lacks this dual functionality. However, the high cost associated with MLPA can render it impractical in resource-limited settings [57, 58]. Despite these challenges, our PCR-based system from DBSc offered a feasible first-line screening method for SMA. It effectively detected *SMN1* deletions, which are a hallmark of the disease. This cost-efficient approach enhances accessibility and facilitates the identification of suspected SMA cases in remote areas, thus preventing diagnosis delay or misdiagnosis.

Conclusion

Our findings showed the potential of our in-house cellulose-based card as an alternative DNA-based carrier and storage for SMA genetic testing. The card's physical characteristics and fiber density are comparable to those of standard cards, making it suitable for molecular analyses. An optimized alternative DBS-carrier might be beneficial for remote SMA diagnosis, particularly in resource-limited settings. However, further studies need to be conducted to evaluate the performance of DBSc for additional high-throughput methods, copy number analysis, or mutation screening.

Abbreviations

SMA	Spinal muscular atrophy
SMN1	Survival motor neuron 1
SMN2	Survival motor neuron 2
DBS	Dried blood spot
DBSc	Dried blood spots on the cellulose-based card
DBSf	Dried blood spots on the FTA card
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
Multi-ASA-PCR	Multiplex allele-specific amplification-PCR

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12896-024-00938-2.

Supplementary Material 1

Supplementary Material 2

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Author contributions

Y.O.S.W and M.A.R performed and designed the experiments and wrote the manuscript. D.K.N.P. provided resources, analyzed data, and reviewed the manuscript. A.F. supervised the project and reviewed the manuscript. All authors have read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

This study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Medical and Health Research Ethics Committee (MHREC) at the Faculty of Medicine, Nursing, and Public Health, Universitas Gadjah Mada (reference number KE-FK-0467-EC-2023, approved on March 2023). Informed consent was obtained from all the subjects involved in the study. The manuscript did not contain any individual person's data in any form (including any individual details, images or videos).

Competing interests

The authors declare no competing interests.

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