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ORIGINAL ARTICLE

Results of a Pilot External Quality Assessment Scheme for Genetic Testing of Newborns with Spinal Muscular Atrophy

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SUMMARY

Background: **This study aims to evaluate the ability of laboratories to perform spinal muscular atrophy (SMA) genetic testing in newborns based on dried blood spot (DBS) samples, and to provide reference data and advance preparation for establishing the pilot external quality assessment (EQA) scheme for SMA genetic testing of newborns in China.**

Methods: **The pilot EQA scheme contents and evaluation principles of this project were designed by National Center for Clinical Laboratories (NCCL), National Health Commission. Two surveys were carried out in 2022, and 5 batches of blood spots were submitted to the participating laboratory each time. All participating laboratories conducted testing upon receiving samples, and test results were submitted to NCCL within the specified date.**
Results: **The return rates were 75.0% (21/28) and 95.2% (20/21) in the first and second surveys, respectively. The total return rate of the two examinations was 83.7% (41/49). Nineteen laboratories (19/21, 90.5%) had a full score passing on the first survey, while in the second survey twenty laboratories (20/20, 100%) scored full.**
Conclusions: **This pilot EQA survey provides a preliminary understanding of the capability of SMA genetic testing for newborns across laboratories in China. A few laboratories had technical or operational problems in testing. It is, therefore, of importance to strengthen laboratory management and to improve testing capacity for the establishment of a national EQA scheme for newborn SMA genetic testing.**
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KEYWORDS

external quality assessment, genetic testing, spinal muscular atrophy, newborn screening

INTRODUCTION

Spinal muscular atrophy (SMA) is an inherited neuromuscular disease characterized by severe disability and high fatality rates. It is the leading cause of death in children under 2 years of age. The main clinical features are muscle weakness and atrophy caused by degeneration and loss of α-motor neurons in the anterior horn of the spinal cord [1-3]. Survival of motor neuron 1 (*SMN1*) is the most predominant causative gene of SMA. About 95% of SMA patients have a homozygous deletion of *SMN1* exon 7, and about 5% have a compound heterozygous mutation of *SMN1* [2-4]. Survival of motor neuron 2 (*SMN2*) is a SMA modifier gene highly homologous to *SMN1*. It can partially compensate for the loss of function caused by *SMN1* mutation and rescue the SMA disease phenotype. The severity of the phenotype in patients with SMA is negatively correlated with their *SMN2* gene copy number. Recently, disease modification therapy targeting *SMN2* and *SMN1* gene replacement drugs to increase the content of SMN protein with complete function has been applied in clinical practice.

Genetic screening of newborns is very important for diagnosing and treating SMA. On the one hand, by screening the copy number of exon 7 of *SMN1*, homozygous deletion variants (copy number of 0) and heterozygous deletion variants (copy number of 1) can be effectively detected, achieving early screening, early diagnosis, and early intervention of SMA, which is of great significance for reducing the mortality of children with SMA. On the other hand, quantitative analysis of *SMN2* gene copy number in newborns with homozygous deletion of *SMN1* can predict the severity of their SMA phenotype to a certain extent, thus providing a reference for the treatment, clinical management, and prognosis evaluation of patients after diagnosis [2,3].

In order to investigate the experimental quality of SMA genetic testing in newborns across domestic laborato-

ries, this survey was designed and carried out by the National Center for Clinical Laboratories (NCCL), hoping to identify the problems in the testing and to prepare for the establishment of external quality assessment and proficiency testing (EQA/PT).

MATERIALS AND METHODS

Survey respondents

Domestic laboratories that carried out newborn SMA genetic testing projects and were willing to participate in the present study were selected as survey subjects. To guarantee the confidentiality of the test results from participating laboratories, each participating laboratory in this project was represented by a unique batch.

Quality control dried blood spots

Quality control dried blood spot (DBS) preparation was as follows: about 45 μL of blood samples were obtained with a pipette and naturally dropped into the filter paper sampling circle. Each blood spot was 12 mm in diameter. A technician was to ensure that both sides of the filter paper were uniform and fully infiltrated. The filter paper of the dried blood sample was successfully prepared after drying at room temperature for 4 hours. Each individual piece of dried blood was placed in a zipped-off bag and independently stored under airtight conditions. In each survey, five batches of quality control DBS samples were selected by investigators of the NCCL. Five quality control filter papers of DBSs with a diameter of 12 mm were distributed to each participating laboratory, including three samples with 0 copies of *SMN1*, one sample with 1 copy of *SMN1*, and one sample with 2 copies of *SMN1*. The sample batch numbers for the first survey were 202211, 202212, 202213, 202214, and 202215 (of which 202211 and 202215 were collected from the same patient). The sample batch numbers for the second survey were 202221, 202222, 202223, 202224, and 202225 (of which 202223 and 202225 were collected from the same patient). The quality control DBS samples were tightly stored at 4℃ with an expiration date of 12 months.

Shenzhen Aone Medical Laboratory prepared and provided the quality control DBS samples used in this investigation. In addition, informed consent was obtained from all guardians of participants.

Evaluation items

The ability of each participating laboratory to perform the copy number of exon 7 of *SMN1* and *SMN2* based on DBS specimens was investigated. Laboratories were required to assay on their own the copy number of *SMN1* in samples. Samples with homozygous *SMN1* deletions were subjected to further *SMN2* gene copy number analysis. Laboratory reports were required to include: 1) the copy number of *SMN1* exon 7, and 2) the results of *SMN2* gene exon 7 copy number analysis of *SMN1* samples with 0 copy number. Each laboratory was asked to report the results within the specified time.

Principle of evaluation

The final score of each batch was comprehensively evaluated based on the accuracy of *SMN1* and *SMN2* genetic testing results, where 14 points were assigned for the correct *SMN1* gene copy number, and 10 points were assigned for the correct *SMN2* gene copy number. The total score of each survey was 100 points, and the full score was considered a survey pass. Gene copy numbers and specific scores for each sample are shown in Table 1.

RESULTS

Regional distribution of the laboratories

A total of 34 laboratories from 14 provinces (municipalities and autonomous regions) were included in the two surveys. The regional distribution of participating laboratories is shown in Table 2 and Figure 1D. Among them, 28 laboratories participated in the first survey, and 21 laboratories participated in the second survey, resulting in return rates of 75.0% (21/28) and 95.2% (20/21), respectively (Figure 1A). The overall return rate of the two surveys was 83.7% (41/49). The laboratories from Henan, Jiangxi, and Liaoning provinces failed to return the results in the first survey and did not sign up for the second survey. In the second survey, the laboratories in Fujian and Jilin provinces were newly added, and the addition of the laboratories in Jilin province filled the vacancy of results in Northeast China. Two surveys finally yielded findings from 10 provinces (municipalities and autonomous regions), covering six out of the seven major geographical divisions in mainland China, and representatively, reflecting the current level of genetic testing for SMA in China based on DBS samples.

A total of 8 laboratories failed to return the results of the two surveys, mainly due to the following reasons: 1) they were affected by the outbreak of the COVID-19 epidemic, and thus, could not return the results within the specified time limit (4/8, 50.0%), and 2) the copy number analysis ability of SMA gene was not available to them (4/8, 50.0%).

Detection methods, platforms, and reagents

SMN1 gene copy number analysis

All 21 laboratories used real-time fluorescence quantitative Polymerase Chain Reaction (RT-qPCR) for *SMN1* exon 7 copy number analysis in the first survey, and qPCR instruments from Shanghai Hongshi Technology Company. 19 laboratories used the *SMN1* detection kit (PCR-fluorescence probe method) from Shenzhen Huizhong Biotech Company, whereas 2 laboratories used the *SMN1* exon deletion detection kit (fluorescence quantitative PCR method) from Shanghai Medicore Technology Company.

Table 1. Scoring scheme for the EQA of SMA genetic testing in 2022.

Survey	Batch number of quality control blood spot	*SMN1* copy number	*SMN1*	*SMN2* copy number	*SMN2*	Score
The first time	202211	0	14’	2	10’	24’
	202212	0	14’	3	10’	24’
	202213	1	14’	/	/	14’
	202214	2	14’	/	/	14’
	202215	0	14’	2	10’	24’
	Total score					100’
The second time	202221	2	14’	/	/	14’
	202222	1	14’	/	/	14’
	202223	0	14’	3	10’	24’
	202224	0	14’	2	10’	24’
	202225	0	14’	3	10’	24’
	Total score					100’

If the *SMN1* score was 0, the *SMN2* score was directly counted as 0.

Table 2. Distribution of participating laboratories.

Region	Province	Number of Labs	
		The first time	The second time
East China	Shandong	4	3
	Jiangsu	2	2
	Fujian	0	1
South China	Guangdong	5	2
North China	Shanxi	3	3
	Inner Mongolia	1	1
	Henan	1	0
Central China	Hunan	2	2
	Jiangxi	1	0
Southwest China	Chongqing	3	2
	Guizhou	1	1
	Sichuan	4	3
Northeast China	Liaoning	1	0
	Jilin	0	1
Total		28	21

A total of 20 laboratories participated in the second survey. All of them used the *SMN1* detection kit (PCR-fluorescence probe method) to analyze the copy number of *SMN1* gene exon 7 by qPCR (Figure 1B).

SMN2 gene copy number analysis

Among the 21 laboratories that participated in the first survey, 19 laboratories used the qPCR method and self-prepared reagents to analyze the copy number of *SMN2* exon 7. All the laboratories used the qPCR instrument from Shanghai Hongshi Technology Company. In addition, two laboratories used the Multiplex Ligation-dependent Probe Amplification (MLPA) method with the probemix p060 SMA detection reagent from MRC-Holland Company. Also, the ABI3500DX sequencing instrument was used.

Table 3. Results of *SMN* gene copy number detection of each sample.

Survey	Batch Number	Correct rate of copy number analysis	
		SMN1	***SMN2***
The first time	**202211 (202215)**	**100% (42/42)**	**95.2% (40/42)**
	202212	**100% (21/21)**	**100% (21/21)**
	202213	**100% (21/21)**	**/**
	202214	**100% (21/21)**	**/**
The second time	**202221**	**100% (20/20)**	**/**
	202222	**100% (20/20)**	**/**
	202223 (202225)	**100% (40/40)**	**100% (40/40)**
	202224	**100% (20/20)**	**100% (20/20)**

Among the 20 laboratories that participated in the second survey, all of them used the qPCR method and self-prepared reagents to analyze the copy number of *SMN2* gene exon 7 (Figure 1C), as well as qPCR instrument from Shanghai Hongshi Technology Company.

Analysis results

Some laboratories participating in this study (11.8%, 4/34) could not do *SMN* gene copy number analysis. Most of the laboratories that successfully reported the results used the qPCR method, and a few used the MLPA method.

The results of the two surveys are shown in Table 3 and Figure 2A. In the first survey, all 21 laboratories used the qPCR method to analyze the copy number of *SMN1* gene exon 7, and the results were all correct. The results of *SMN2* gene exon 7 copy number analysis in 19 laboratories using the qPCR method and self-prepared reagents were also all correct; however, in two laboratories using the MLPA method, the reported analysis results of the *SMN2* gene exon 7 copy number in the quality control blood spot of batch 202215 were not consistent with the expected results. In the second survey, 20 laboratories used the qPCR method for *SMN1* and *SMN2* gene copy number analysis, achieving an accuracy of 100% on the returned results. The specific scores of each laboratory are shown in Supplemental Tables 1, Supplemental Tables 2, and Figure 2B, C. Among the 34 laboratories participating in this study, 15 participated in both surveys (see Supplemental Figure 1 for the participation of each laboratory). These laboratories used the same detection method in the two surveys, and all obtained full marks, indicating stable and reliable SMA detection capability.

DISCUSSION

In the first survey, 5 batches of quality control blood spots were distributed, and 21 out of 28 participating laboratories reported the results. Among them, 19 laboratories (19/21, 90.5%) passed the survey with full marks, while 2 laboratories (2/21, 9.5%) failed to obtain full marks as the analysis results of the number of copies of *SMN2* gene exon 7 of the quality control spots of the batch number 202215 were inconsistent with the expected results. In the second survey, 5 batches of quality control spots were distributed to 21 laboratories, 20 of which reported the results. All 20 laboratories passed the survey with full marks (pass rate of 100%).

qPCR and MLPA are two commonly used methods for copy number analysis among SMA-related genetic testing technologies. The MLPA method uses a hybridization probe for the base difference site in exon 7 of *SMN1* and *SMN2*, where the copy number of *SMN1* and *SMN2* can be simultaneously and directly detected using multiple housekeeping genes as an internal reference. "Expert consensus on newborn screening for spinal muscular atrophy" clearly points out that MLPA is the gold standard for SMA diagnosis recommended by the current SMA management consensus at home and abroad [5]; however, it is not suitable for large-scale screening due to its relatively complex operation, long detection time, low throughput, and high price [6,7]. Nevertheless, the two laboratories that failed to pass the first survey with the full score used this method for *SMN2* gene exon 7 copy number analysis, failing to analyze the *SMN2* gene copy number of sample 202215 correctly. It is possible that the MLPA method has high requirements for the quality of DNA samples, and the operator needs to measure the DNA concentration accurately. Moreover, the operation process is relatively complex, and the samples can be easily contaminated. Since the two quality control blood spot samples with batch numbers 202211 and 202215 were collected from the same patient, and the results of the two laboratories for batch number 202211 were correct, the possibility of sample contamination or inter-sample confounding could not be excluded.

Currently, qPCR is the mainstream technology used for SMA screening in newborns at home and abroad. It is easy to operate, has rapid detection ability, high

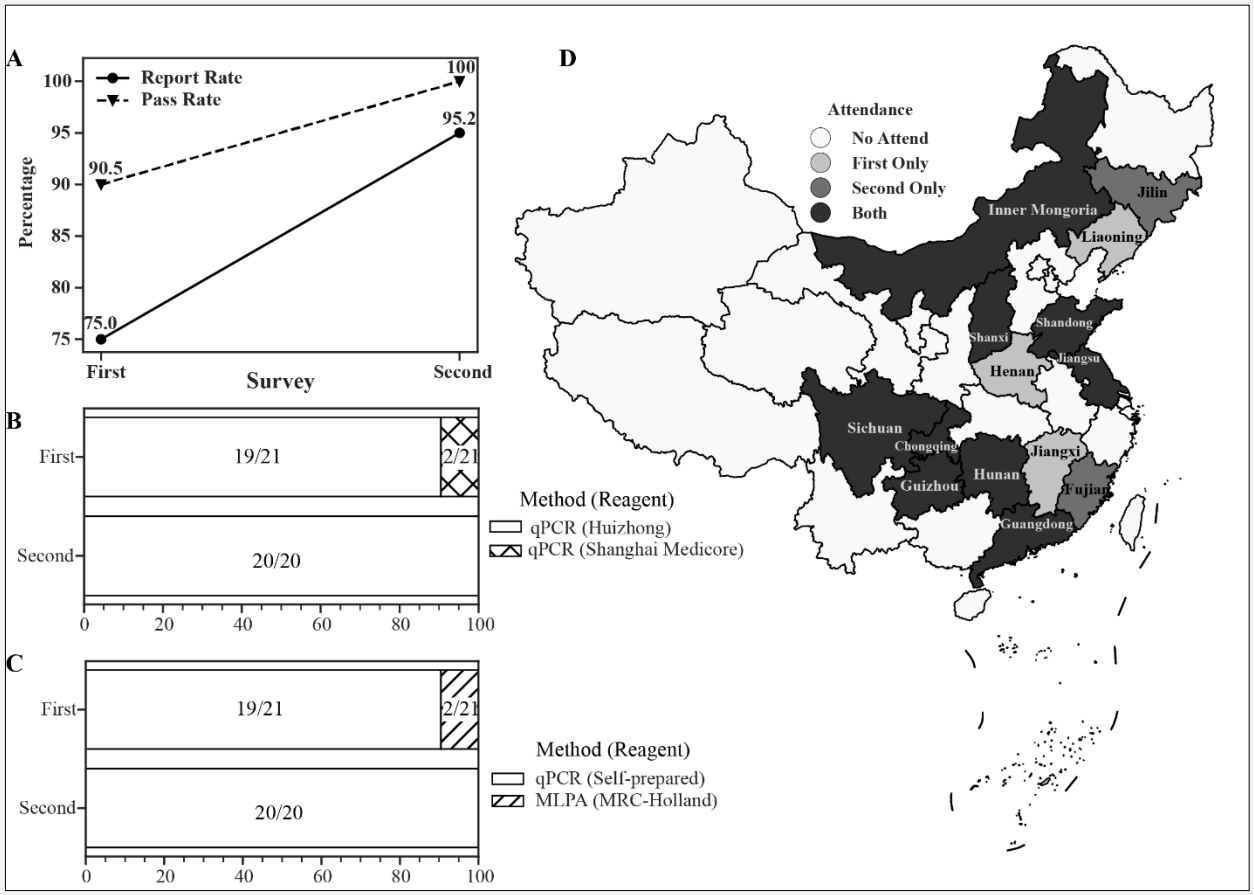

Figure 1. Summary of the result of the pilot EQA scheme.

A - The return rate of survey results. The return rate of experimental results is represented by solid dots and connected by solid lines. The return rate of the first survey is 75.0%, and of the second survey is 95.2%. The pass rate is represented by a solid triangle and connected by a dashed line, with a pass rate of 90.5% for the first survey and 100% for the second survey.
B - Methods and reagents for *SMN1* gene exon 7 copy number analysis in the two surveys. All laboratories used the qPCR method for *SMN1* gene copy number detection. In the first survey, 19 laboratories used the kit from Shenzhen Huizhong Biotech Company, and 2 laboratories used the kit from Shanghai Medicore Technology Company. In the second survey, all 20 laboratories used the kit from Shenzhen Huizhong Biotech Company. Huizhong: Shenzhen Huizhong Biotech Company; Shanghai Medicore: Shanghai Medicore Technology Company.
C - Methods and reagents for *SMN2* gene exon 7 copy number analysis in the two surveys. In the first survey, 19 laboratories used the qPCR method and self-made reagents for *SMN2* gene copy number detection, and 2 laboratories used the MLPA method and MRC-Holland reagents. In the second experiment, all 20 laboratories used the qPCR method and self-prepared reagents.
D - Regional distribution of participating laboratories. Colors from light to dark indicate not participating (white), participating only in the first survey (light grey), participating only in the second survey (grey), and participating in both surveys (black), respectively. A total of 14 provinces (municipalities/autonomous regions) were enrolled in the survey, of which 9 laboratories participated in both surveys, 3 laboratories only participated in the first survey, and 2 laboratories only participated in the second survey.

throughput, and cost-effectiveness, and is suitable for large-scale population screening [8,9]. The qPCR fluorescence probe method uses multiple PCR reactions, housekeeper gene sequence as the internal reference, and fluorescence probe to conduct a semi-quantitative analysis of *SMN1* or *SMN2* gene exon 7 to determine its copy number. At present, *SMN1* gene detection kits from different manufacturers in China have been approved by the National Food and Drug Administration (NMPA) Class III registration certificate. In the first survey, 90.5% (19/21) of the laboratories used the *SMN1* detection kit (PCR-fluorescence probe method) (National Instrument Registration No. 20223400080). In addition, 9.5% (2/21) of the laboratories opted for the

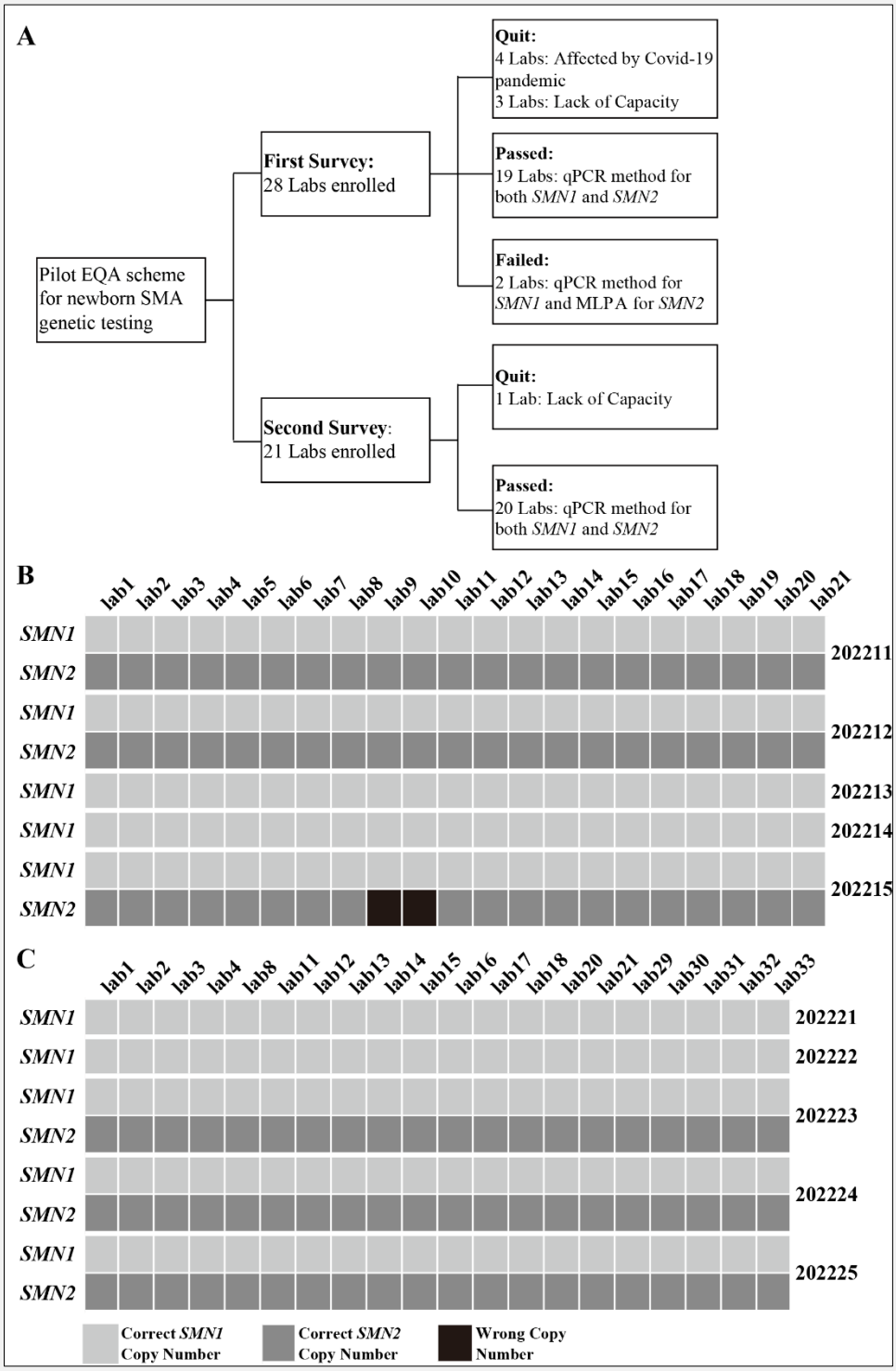

Figure 2. A - Summary of the results of the two surveys - Of the 28 laboratories enrolled in the first survey, 7 quit for Covid-19 pandemic or lack of capacity, 19 passed the assessment, and 2 failed. Of the 21 laboratories enrolled in the second survey, 1 quit for lack of capacity, and 20 passed.
B & C - Quality control blood spot scores of all batches. Light grey - *SMN1* gene copy number analysis was correct, Grey - *SMN2* gene copy number analysis was correct, Black - copy number analysis results were incorrect. In the first survey, the *SMN2* gene copy number analysis results of batch sample 202,215 returned by lab9 and lab10 were wrong, and the full score was not obtained. All other laboratories that submitted results passed with full marks.

SMN1 test kit from Shanghai Medicore Technology Company (National Instrument Registration No. 20223 400080). From the prospective use of this kit, it was only used for the qualitative detection of homozygous deletion of the *SMN1* gene, while the kit from Shenzhen Huizhong Biotech Company was used for copy number variation analysis, which could detect homozygous deletion type, heterozygous deletion type, and normal type of *SMN1* gene. The detection ability of the three *SMN1* genotypes mentioned above should be simultaneously considered in newborn SMA screening. In the second survey, all participating laboratories performed *SMN1* and *SMN2* gene exon 7 copy number analysis using qPCR. Although the specificity of the qPCR method was not as good as MLPA, it is more suitable for large-scale population screening due to its easy operation and low cost. All the 20 laboratories adopted the *SMN1* detection kit (PCR-fluorescent probe method), achieving correct testing results, which may be due to the following reasons: first, the minimum detection limit of the kit for genomic DNA is 2 ng/μL, and only a small number of DBSs is needed to meet the requirements; second, the kit uses the fluorescence probe method, with the probe possibly increasing the specificity of the collected signal as only amplifying the probe binding fragment can collect the signal, and thus effectively block the interference of *SMN2*; and third, the kit is equipped with a UNG enzyme anti-contamination system, which can reduce non-specific PCR amplification and contamination, and ensure the specificity and accuracy of amplification results. Furthermore, qPCR is used to detect the *SMN2* gene, which can ensure the stability and reliability of the overall detection results. So far, no qPCR *SMN2* gene detection kit on the market has received an NMPA Class III registration certificate. Therefore, all laboratories used self-prepared reagents for *SMN2* gene copy number analysis based on qPCR methods.

The type of quality control sample used in this survey was a dry blood spot, and only one dry blood spot with a diameter of 12 mm was provided for each batch number. To simultaneously complete the copy number analysis of *SMN1* and *SMN2*, relatively high requirements were put forward for participating laboratories. Due to the convenience of sample collection, preparation, transportation, and preservation, the DBS sample was the most favorable sampling method for newborn SMA gene screening [5]. Therefore, expanding the SMA screening project under the current newborn screening system without increasing the burden of clinical sampling depends on whether a simple, rapid, accurate, and cost-effective method can be established for automated genomic DNA extraction and genetic testing from DBSs based on a small amount of dry blood spot samples. At present, it is known that areas of DBSs used in the test vary among laboratories. Some laboratories use one complete dry blood spot with a diameter of 12 mm; some use 3-5 DBSs with a diameter of 3 mm, and some use 2 DBSs with a diameter of 6 mm. Yet, some laboratories only use a DBS with a diameter of 4 mm [10]. Laboratories are encouraged to optimize the testing methods to reduce the use area of DBSs and to ensure testing quality.

In addition, the two surveys set different time limits for reporting results, with 30 days for the first survey and 15 days for the second. However, our results showed that all laboratories with copy number testing ability reported results within the specified time limit, thus providing a reference for setting the reporting time limit of the EQA scheme in the future.

The tertiary prevention of SMA is the future development direction. The marketing of SMA therapeutic drugs and their introduction into national medical insurance can also promote SMA screening in newborns [11]. Clinical trials have confirmed that patients who start treatment before the onset of symptoms have greater benefits in terms of survival rate and achievement of movement milestones. "Early treatment, especially treatment before the onset of disease" is of utmost importance. Newborn screening provides a golden opportunity for SMA treatment, which is essential to ensure early diagnosis and early treatment of SMA and to obtain the best prognosis. Some developed countries and regions have accumulated practical experience in SMA screening and testing and have established a complete implementation plan from screening to diagnosis to genetic counseling [12-15]. In China, various provinces and regions have also been gradually carrying out newborn SMA genetic testing projects. Over 10% of newborn screening centers nationwide participated in this pilot EQA scheme. Thus, the result of the survey provided a preliminary understanding of the laboratory's SMA gene detection ability based on DBS samples. It was revealed that some laboratories may have problems with detection technology, experimental operation, sample processing, and other aspects. Furthermore, the establishment of a national EQA scheme system for newborn SMA genetic testing is of great significance for strengthening laboratory management, standardizing testing processes, improving detection capability, as well as improving the level and quality of newborn SMA screening in China [16]. We will involve more participants in the future EQA scheme to provide a broad basis for the quality control of newborn SMA gene testing.

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Data Availability Statement:
All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethical Approval:
The local Institutional Review Board deemed the study exempt from review.

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Declaration of Interest:
The authors declare that they have no conflicts of interest regarding the publication of this work. No financial or non-financial benefits have been received or will be received from any party related directly or indirectly to the subject of this article.

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