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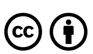
High Throughput Newborn Screening for Sickle Cell Disease – Application of Two-Tiered Testing with a qPCR-Based Primary screen

Hochdurchsatz-Neugeborenenscreening auf Sichelzellkrankheit – Anwendung einer zweistufigen Analytik mit einem qPCR-basierten Primärscreening



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ABSTRACT

Background Sickle cell disease (SCD) is a group of hemoglobinopathies with a common point mutation causing the production of sickle cell hemoglobin (HbS). In high-throughput newborn screening (NBS) for SCD, a two-step procedure is suitable, in which qPCR first pre-selects relevant samples that are differentiated by a second method.

Methods Three NBS centers using qPCR-based primary screening for SCD performed a laboratory comparison. Methods using tandem MS or HPLC were used for differentiation.

Results In a benchmarking test, 450 dried blood samples were analyzed. Samples containing HbS were detected as reliably by qPCR as by methods established for hemoglobinopathy testing. In a two-step screening approach, the 2nd-tier-analyses have to distinguish the carrier status from pathological variants. In nine months of regular screening, a total of 353,219 samples were analyzed using two-stage NBS procedures. The 1st-tier screening by qPCR reduced the number of samples for subsequent differentiation by > 99.5 %. Cases with carrier status or other variants were identified as inconspicuous while 78 cases

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with SCD were revealed. The derived incidence of 1:4,773, is in good agreement with previously published incidences.

Conclusion In high-throughput NBS for SCD, qPCR is suitable to focus 2nd-tier analyses on samples containing HbS, while being unaffected by factors such as prematurity or transfusions. The substantial reduction of samples numbers positively impacts resource conservation, sustainability, and cost-effectiveness. No false negative cases came to attention.

ZUSAMMENFASSUNG

Hintergrund Die Sichelzellkrankheit (SCD) bezeichnet eine Gruppe von Hämoglobinopathien mit einer gemeinsamen Punktmutation, die zur Bildung von Sichelzell-Hämoglobin (HbS) führt. Für das Hochdurchsatz-Neugeborenenscreening (NGS) auf SCD bietet sich ein zweistufiges Verfahren an, in dem die qPCR HbS-haltige Proben vorselektiert, die mit einer zweiten Methode differenziert werden.

Methoden Drei NGS-Zentren, in denen ein qPCR-basiertes Primärscreening auf SCD durchgeführt wird, haben sich einem Laborvergleich unterzogen. Zur Differenzierung wurden Tandem-MS oder HPLC genutzt.

Ergebnisse In einem Laborvergleich mit 450 Trockenblutproben wurden HbS-haltige Proben mit qPCR ebenso zuverlässig erkannt, wie mit Methoden die zur Untersuchung von Hämoglobinopathien etabliert sind. Der Fokus der Folgeanalytik liegt beim zweistufigen SCD Screening somit auf der Unterscheidung zwischen Trägerstatus und pathologischen Varianten. In neun Monaten Regelscreening wurden insgesamt 353.219 Proben untersucht, wobei das 1st-tier-NGS mittels qPCR die Probenzahl für die Differenzierung um > 99,5 % reduzierte. Fälle mit Trägerstatus oder andere Varianten wurden als unauffällig erkannt und 78 Fälle mit SCD diagnostiziert. Die abgeleitete Inzidenz von 1:4.773, stimmt gut mit bislang publizierten Inzidenzen überein.

Schlussfolgerung Im Hochdurchsatz-NGS auf SCD ist qPCR geeignet, um die Folgeanalytik auf Proben zu fokussieren, die HbS enthalten und dabei von Störkonstellationen wie Frühgeburtlichkeit oder Transfusionen unbeeinflusst zu sein. Die erhebliche Reduzierung der Probenzahl wirkt sich positiv auf Ressourcenschonung, Nachhaltigkeit und Wirtschaftlichkeit aus. Falsch negative Befunde sind nicht bekannt geworden.

Introduction

Sickle cell disease (SCD) is a serious disease leading to circulatory disorders, organ damage, severe pain and early death [1]. According to current figures, about 400,000 are born with SCD every year [2]. The term SCD comprises a group of hemoglobin (Hb) disorders, which are autosomal recessively inherited and characterized by the presence of hemoglobin S (HbS) resulting from a point mutation in the 6th codon of the β-globin encoding *HBB* gene (*HBB* CD 6 GAG > GTG [Glu > Val]). The underlying genotype of the disorder can be homozygous or compound-heterozygous in case a certain second pathogenic *HBB* mutation is present. In the deoxygenated form, HbS tends to polymerize, resulting in the eponymous sickle shape of affected erythrocytes, which are prone to aggregation and hemolysis [3, 4]. In newborns, fetal hemoglobin (HbF, $\alpha_2\gamma_2$) represents the largest Hb share in the child's blood with HbA accounting for approx. 1/4 at term. During the first months of life, the HbA concentration continues to increase. After reaching a critical level, which typically occurs between the third and fourth month, symptoms begin to manifest in SCD-affected individuals, commonly within the first year of life [5]. The clinical presentations of such persons include acute pain, vaso-occlusions, and chronic organ complications with the severity of symptoms differing among SCD variants. [3, 4]. Individuals with sickle cell trait (HbS/A), have only one mutated *HBB* allele and do not develop symptoms. Early detection of SCD patients has proven to be beneficial for disease management, for treatment outcome, and substantially reduces morbidity and mortality [5, 6]. Thus, SCD has been included as target disease in newborn screening (NBS) programs in several countries; in Europe, these are Belgium, France, Malta, the Netherlands, the United Kingdom, Spain, and, since October 2021, Germany [7, 8].

Due to the Hb variants relevant to SCD, screening methods used for its detection usually rely on the analysis of corresponding proteins or peptides and include high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), isoelectric focusing, MALDI-TOF or tandem mass spectrometry (MS/MS) [7, 9]. For NBS laboratories, inclusion of new target diseases in existing screening programs can be challenging in many aspects, e. g., considering sample logistics, spatial, personnel, and analytical capacities, particularly if they operate in high-throughput conditions (e. g., > 1,000 samples per day). Hence, methods are required that do not noticeably affect the throughput rate, are cost-efficient and meet analytical specifications, such as sufficient sensitivity [8]. Apart from investing in new instruments, strategies such as multiplexing or the implementation of multi stage analytical processes are applied to address such challenges; in the first case, new targets are integrated into existing methods, platforms or workflows, in the latter case, a 1st-tier method performs the mass-screening and one or more subsequent methods with comparably higher specificity than the first one are used for confirmation and differentiation [10, 11].

Of the mentioned techniques applied to SCD screening, only MS/MS has been commonly used in German NBS laboratories. However, the required sample preparation is incompatible with existing NBS methods (i. e., analysis of acylcarnitines and amino acids) and cannot be integrated in existing workflows [12]. Since the inclusion of severe combined immunodeficiencies (SCID) as target disease in 2019 and spinal muscular atrophy (SMA) in 2021, concurrent with SCD screening, qPCR is present in German NBS laboratories. This analytical platform is capable of multiplexing [13], and specific PCR-based methods to detect HbS alleles have been published [14]. As SCD arises from a nucleotide change present in

all its forms, qPCR is a predestined method to perform the primary screening in a two-tiered analytical approach and preselect HbS-containing specimens. A subsequent 2nd-tier method is then needed to distinguish HbS/A cases from specimens with Hb variants indicative of SCD. Such screening approaches have been developed and applied in three high-throughput NBS laboratories, which together account for over 60 % of the German NBS capacity [15]. In a joint effort, an extensive sample exchange was set up pursuing two goals: i) As a quality assurance task for the screening processes and ii) to demonstrate that a two-step analytical approach with a qPCR-based primary screening is suitable to reliably detect SCD cases. Here, the results of these benchmarking tests are presented together with a summarized outcome of the first nine months of regular SCD screening in these laboratories.

Materials and Methods

Samples

Most patient samples for benchmarking were taken from Guthrie-cards sent to the laboratories for regular NBS and for which written informed consent was given. In addition to the NBS specimens, several previously donated and anonymized, pathologic samples were included for which written consent was given. Each of the three laboratories compiled three identical sets of 150 dried blood spot (DBS) samples, that were anonymized and double blinded. The aim was to detect and specify a small proportion of SCD-relevant samples and a larger proportion of heterozygous (S/A) among predominantly wild-type samples, without considering a distribution of phenotypes representative for the German population in the compilation. One set each was sent to the other laboratories.

To compare the results of the individual analytical platforms, the benchmarking sample sets were entirely analyzed, i. e., using all analytical methods that are part of the laboratories' SCD screening. In the regular NBS, however, the methods were applied in the conceptualized, two-tiered analytical procedures, so that the methods for differentiation were used only on samples that had been preselected by qPCR.

Analytical methods

Each of the three laboratories' screening approaches is based on two-tier testing procedures using a multiplex qPCR method for mass screening that allows detection of SCID and SMA in parallel with HbS alleles. Subsequent 2nd-tier methods are used to differentiate the pre-selected subset of samples.

In Lab 1 and Lab 2, the qPCR analyses are performed using Lightmix kits (TIB Molbiol, Berlin, Germany) and a Lightcycler 480 System (Roche Diagnostics International, Rotkreuz, Switzerland) with high-resolution melting curve analysis to detect *HBB* variants. In Lab 3, an in-house developed qPCR method using specific fluorescent TaqMan probes (synthesized by Integrated DNA Technologies, Coralville, USA) is performed using a Quantstudio 7 Flex System (Applied Biosystems, Massachusetts USA).

For differentiation of the preselected specimens, automated HPLC systems were used in Lab 1 (Variant nbs, Bio-Rad, Feldkirchen, Germany) and Lab 2 (HLC-723G8, TOSOH, Stuttgart, Germany), which were operated according to the manufacturers' instructions. Lab 3 used Newborn haemoglobinopathy screening kits (SpotOn Clinical Diagnostics Ltd., London, Great Britain) with a modified protocol in combination with a flow injection analysis MS/MS system (Acquity UPLC and Xevo TQD, Waters, Eschborn, Germany) for this task. At the time of the benchmarking tests, a CE system (Capillarys 3 DBS, Sebia, Lisses, France) was evaluated in Lab 1 for differentiation, so that results for these samples could also be generated with this system, even if it was not subsequently applied as part of the lab's two-tiered approach.

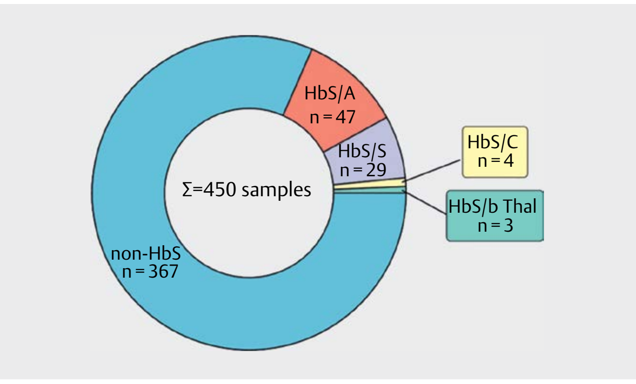
More detailed descriptions about the methods, including measures for quality control, are provided in the supplemental file (section "Method descriptions") and elsewhere [13, 16].

Results

Benchmarking

Three equal sets of 150 DBS samples were provided by each of the three laboratories and distributed among them to be analyzed. The composition of genotypes in these 450 benchmarking samples is shown in ▶ **Fig. 1**. In total, 36 samples of these represented physiological conditions considered positive in an SCD screening setting: 29 homozygous HbS/S, four compound-heterozygous HbS/C, and three HbS/β thalassemia (HbS/βThal, no further characterization available). Forty-seven specimens were of sickle cell trait, and the 367 specimens designated as "non-HbS" were of Hb types that did not contain HbS (mostly wildtype, four HbC/A samples and one HbC/C).

All benchmarking samples were analyzed by means of both methods used for SCD screening in the labs, i. e., three qPCR, two HPLC methods, and one MS/MS method. In addition, Lab 1 provided results obtained with CE for these samples although this platform has not been applied in its regular SCD screening. The results are summarized in ▶ **Tab. 1**. A detailed list of the individual results is given in the **supplemental file (Tab. S 1)**. Among the 450 samples, 36 belong to genotypes relevant for SCD disease, and 414 are considered negative (367 non-HbS and 47 HbS/A) in an NBS scenario. However, in a two-step screening process with an integrated preselection of HbS-containing samples in the 1st tier (i. e., based



▶ **Fig.1** Distribution of genotypes in the total benchmarking sample set. The term 'non-HbS' here refers to variants devoid of HbS, i. e., mainly wildtype; the figure was created with R (4.1.2) and ggplot2 (3.3.5).

▶ **Tab. 1** Numbers (*n*) of specimens per type included in the benchmarking samples and returned results by applying the respective laboratories' individual 1st–tier PCR– and their 2nd–tier differentiation methods and CE. The full set of samples has been analyzed by each method. For PCR–based methods, a further differentiation of HbS containing samples is not pursued. *CE results were provided by Lab 1 although this platform is not part of the two–tiered analytical setups. **HbS/βThal: compound heterozygous HbS/β thalassemia.

		Lab 1		Lab 2		Lab 3		
Type	***n***	**PCR**	**HPLC**	**PCR**	**HPLC**	**PCR**	**MS/MS**	**CE***
non–HbS	367	362	367	362	367	367	367	367
HbS–containing	83	88	83	88	83	83	83	83
HbS/A	47	–	47	–	47	–	47	47
HbS/S	29	–	32	–	31	–	31	30
HbS/C	4	–	4	–	4	–	4	4
HbS/βThal**	3	–	0	–	1	–	1	2

▶ **Tab. 2** Individual numbers of screened samples, suspect and reported SCD findings of the individual labs and the respective sums. Other SCD variants in scope of the screening, such as HbS/D, HbS/E, or HbS/O were not detected.

	Lab 1	Lab 2	Lab 3	Sum
total samples screened	**129,194**	**120,009**	**104,016**	**353,219**
samples flagged for 2nd tier	613	591	430	1,634
SCD positive	**31**	**23**	**24**	**78**
HbS/S	13	19	16	48
HbS/C	3	2	5	10
HbS/β0-Thal	1	1	2	4
HbS/β+-Thal	14	1	0	15
HbS/HPFH	–	–	1	1

on qPCR targeting HbS alleles), it is equally necessary to recognize the 47 HbS/A samples.

In the sample exchange, the 83 samples containing HbS were found by all methods applied. However, in the qPCR tests, Lab 1 and Lab 2 categorized five additional samples as "HbS-containing" ($\Sigma = 88$) which turned out to be of those genotypes containing HbC after unblinding. In the form used to submit the test results, these five samples were annotated in the 1st-tier method stating that "HbC could not be excluded". As expected, application of the respective methods intended for differentiation not only allowed to distinguish between "non-HbS" and the "HbS-containing" sample groups, but also to further elucidate the latter: All 47 specimens of HbS/A were correctly recognized and SCD-variant specimens categorized. However, while homozygous HbS/S and compound-heterozygous HbS/C type samples were identified, one or more of the HbS/βThal samples were labelled HbS/S using either of the three methods, CE, HPLC, or MS/MS.

Regular screening

As of October 1st, 2021, SCD was included in the German NBS panel as new target disease. Hence, the two-tiered screening strategies described here were applied in routine NBS from then on. The number of screened specimens in nine months between October 1st, 2021 and June 30th, 2022 and the results of the SCD screening are summarized in ▶ **Tab. 2**.

In the individual labs, 104,016, 120,009, and 129,194 samples have been analyzed during this period accounting for 353,219 samples in total, which went through qPCR-based primary screening. Of these, 1,634 samples (0.46 %) were flagged for subsequent differentiation, thus, the qPCR-based screening reduced the number of samples requiring differentiation by more than 99.5 %. As expected, the lion's share of the specimens preselected by qPCR were of heterozygous HbS/A or non-SCD variants and, as such, classified as screen negative after 2nd-tier analyses. However, during the nine months of routine screening, 78 SCD patients were revealed, reported, and referred for follow-up: In total, 48 homozygous HbS/S cases, ten HbS/C, four HbS/β0 thalassemia, 15 HbS/β+ thalassemia cases, and one child having HbS combined with hereditary persistence of fetal hemoglobin (HPFH). To date, as far as feedback could be obtained through tracking, 59 of the reported cases have been confirmed. Feedback on eight HbS/S, two HbS/C and four HbS/β+ thalassemia findings is still pending. Five of the reported HbS/β+ thalassemia findings have turned out to be heterozygous HbS/A.

Discussion

Benchmarking

In a sample exchange, identical anonymized and double-blinded sets of 450 DBS samples were analyzed at three different sites using one individual qPCR method per lab and different analytical platforms (intended for differentiation) with commercial kits designed for hemoglobinopathy analyses: CE, 2 × HPLC, and MS/MS. After unblinding and comparing the reported results to target conditions, it was found that the "non-HbS" ($n = 367$) and "HbS-containing" ($n = 83$) sample groups were distinctly separated applying CE, HPLC, MS/MS, and TaqMan-based qPCR. Application of two qPCR methods that employed melting curve analysis concordantly resulted in 362 samples categorized as non-HbS ("screen negative"), so that five additional specimens (four HbC/A and one HbC/C) would have been flagged and forwarded to 2nd-tier analysis for differentiation. Both β-globin encoding gene variants (*HBB* c.20 A > T, p.Glu6Val for HbS and *HBB* c.19 G > A, p.Glu6Lys for HbC) are situated on the same codon in the *HBB* gene [3]. In qPCR with melting curve analysis, the signals for both alleles appear at similar T_m values ($\Delta T_m = 1°C$). However, the aim of the applied algorithm in 1st-tier screening is variant detection, thus all non-wildtype samples

are flagged at this stage, independent of the underlying variant. Moreover, the logical consequence for any equivocal results in analytical tests is further clarification, e. g., by repetition with an extended experimental design or by applying an independent method. The latter is the core of two-stage analytical processes, and it is therefore consistent to flag such samples for later verification (or rebuttal) [11]. Here, this is done by HPLC-based differentiation as described in the 'Materials and Methods' section (and the supplemental file). In all cases, the set goal for qPCR, to preselect all specimens containing HbS (i. e., no false-negatives) in the sample exchange, was fully achieved.

The distinction of genotypes in the benchmarking analyses was performed with two independent HPLC methods, one MS/MS, and one CE method. All of these correctly categorized HbS/A, HbS/S, and HbS/C variants. Sickle cell carriers are typically asymptomatic and must not be reported after screening. Even if such samples are preselected in the qPCR 1st tier, it could be demonstrated that the 2nd-tier method clearly recognizes HbS/A specimens (and other non-SCD variants forwarded to differentiation) as screen negatives. For the three compound-heterozygous HbS/β Thal in the benchmarking samples, with each differentiation method, at least one of the specimens was classified as HbS/S. Homozygous HbS/S, HbS/β^0 thalassemia, and HbS/HPFH share the same Hb patterns (F and S) and can thus not be distinguished by means of the mentioned methods. In case of HbS/β^+ thalassemia, HbA additionally appears in the pattern, and depending on the residual expression of ß-globin from the thalassemic *HBB* allele, indicative ratios of calculated variant to wildtype signals can overlap with the ranges of HbS/A [17, 18]. The main objective of NBS programs, however, is to improve outcomes for individuals at risk for a disease through early detection and thus early treatment and care [5]. Moreover, screening results are considered presumptive and are to be confirmed by independent analytical methods in follow-up. Considering the benchmarking results, in a two-stage screening setup of qPCR and one of the applied differentiation methods, neither false positives (non-SCD) nor false negatives would have been reported. The goal of identifying specimens representing affected patients would hence also be fulfilled.

Regular screening

SCD was included as target disease in the German NBS program on October 1st, 2021, so the different combinations of two-tiered approaches and strategies described above were employed in routine screening since then. More specifically, the qPCR-based primary screen was realized within multiplexed methods that enabled the simultaneous detection of SCID, SMA as well as HbS specimens in a high-throughput environment (e. g., > 1,000 samples on peak days). In the nine months ending June 30th, 2022, a total of 353,219 patient samples had passed through the three NBS centers, and 1,634 samples of these were investigated with 2nd-tier analyses. Compared to a setting in which the full number of received samples would have been screened for SCD by HPLC, CE, or MS/MS, the antecedent qPCR step efficiently reduced the number of specimens to be reanalyzed by more than 99.5 %. Of the specimens characterized as non-wildtype or HbS-containing, the majority (1,556 or 95.2 %) was recognized as non-SCD (i. e., HbS/A or a variant not relevant for SCD) during differentiation by means of 2nd-tier HPLC or MS/MS, ensuring these samples were reported as screen negative. In contrast, 78 patients were detected with genotypes consistent with SCD (48 HbS/S, 15 HbS/β^+ thalassemia, ten HbS/C, four HbS/β^0 thalassemia, and one HbS/HPFH). In follow-up, all cases reported as HbS/S, HbS/C, HbS/β^0 thalassemia, or HbS/HPFH and for which feedback could be obtained during tracking were confirmed. However, no results from confirmation diagnostics have been communicated for 14 patients to date. Five of the cases that were reported as HbS/β^+ thalassemia have been found to be HbS carriers without β thalassemia. Due to the overlapping ranges of S/A ratios, the differentiation of HbS/β^+ thalassemia and HbS/A is a known difficulty [17]. Here, the analytical evaluation was designed to avoid false-negatives, which in turn means that some false-positive findings for HbS/β^+ thalassemia may occur.

Assuming that pending confirmation results prove positive, these results correspond to an overall incidence of 1:4,773, which is in good agreement with a statistical evaluation of health insurance data (1:5,102) [19], a previous study in Germany that included urban and rural areas near Berlin (1:4,154) [12], and comprehensibly lower than in two studies representing mainly urban areas (Hamburg: 1:2,385 and Berlin: 1:2,433) [20–22]. Furthermore, after nine months of regular screening (and until the preparation of this manuscript), no false-negative results have been brought to our attention.

Further aspects of qPCR-based two-tiered SCD screening

In routine NBS, two often discussed drawbacks are prematurity of babies and transfusions. In premature children, the share of HbF in the blood highly exceeds the proportion of adult HbA (e. g., probably detectable from 30 weeks gestation and 5–10 % by 34–36 weeks gestation) [5]. Blood transfusions, however, lead to a dilution of the patient's blood and may produce misleading analytical results in case of an SCD patient, e. g., wrong diagnostic ratios indicating sickle cell trait or even a wildtype condition. In contrast to biochemical methods, qPCR targets nucleic acid sequences rather than peptides and is thus intrinsically unaffected by such drawbacks. Hence, even in cases of low concentrations of natively synthesized β-globin molecules, this technique reliably detects HbS alleles, which represents one more advantage in an NBS environment.

As stated above, < 0.5 % of the samples entering the laboratories had to be investigated by 2nd-tier analyses after the qPCR primary screening. Such a drastic reduction in analytical workload is also associated with substantial savings in chemicals, consumables, energy, and working time of specialized personnel, thus enabling economical and more sustainable operation. To highlight this aspect in more detail, exemplary comparisons of consumables and solvents for the described methods applied in different settings are provided in the **supplemental file (Tab. S 2)**. If SCD screening was based solely on MS/MS in Lab 3, 2,100 more microplates, 107,000 additional pipette tips, and 43 L more MeOH would have been spent during the nine-month study period.

According to 2019 data from the Global Burden of Disease project, SCD is responsible for 0.6 deaths, 44.8 years of life lost and 48.7 years lived with disability, each per 100,000 individuals [23]. SCD is widespread in sub-Saharan Africa and central India. In low-

and middle-income countries such as these, it is difficult to raise the necessary funds for expensive ready-to-use newborn screening tests [24], so SCD is often not diagnosed until life-threatening situations occur or severe pain requires a hospitalization of children. In a recent retrospective observational study, the mortality rate for infants under five years of age in sub-Saharan Africa was estimated to be about 36.4 %. [25]. Besides cost, the availability of a detection method is an important factor. During the SARS-CoV 2 pandemic, qPCR has experienced a boost in dissemination, and in light of multiplex testing, it can be a cost-effective and readily available analytical platform for many purposes. While the approved NBS tests for SCD applying HPLC, MS, or CE account for a large part of (routine) Hb variant analyses, tests based on, e. g., enzyme-linked immunoassays, chip microarrays, or other techniques have also been developed and may provide cost-effective alternatives [26]. In order to achieve a pre-selection within a cohort and thus to focus the use of such methods on relevant samples, a combination with qPCR may in turn be reasonable.

Conclusions

In benchmarking tests, it could be demonstrated that qPCR is well suited to detect samples carrying the HbS point mutation and thus to reliably perform a preselection in two-tiered analytical setups for SCD screening. As expected, established analytical platforms were subsequently successful in distinguishing the carrier state from SCD cases and differentiating SCD variants based on the detected Hb patterns. During nine months of regular screening, 353,219 patient samples were analyzed in three high-throughput NBS centers applying two-tiered analytical procedures for SCD screening that incorporated a qPCR-based 1st-tier within multiplexed methods. This preselection step efficiently reduced the sample numbers for the 2nd-tier methods by more than 99.5 % spotlighting specimens relevant for further testing, i. e., non-wildtype or HbS-containing samples, while not being affected by confounding factors such as prematurity or transfusions. Further investigations by HPLC or MS/MS used as 2nd-tier methods ensured that individuals with carrier state or non-target variants were categorized SCD negative, while 78 patients with SCD-variants were revealed: 48 HbS/S, 15 HbS/β^+ thalassemia, ten HbS/C, four HbS/β^0 thalassemia, and one HbS/HPFH. As far as feedback has been received, all of them but four cases of HbS/β^+ thalassemia were confirmed in follow-up, and concurrently, no false-negatives have emerged. Compared to a scenario in which all samples would have been screened with HPLC or MS/MS only, the massive reduction of sample numbers by 1st-tier qPCR testing also resulted in a more cost-effective and sustainable workflow. Combining qPCR with less costly methods for differentiation as the established ones may provide an option for SCD screening to middle- or low-income countries.

Contributor's Statement

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Conflict of Interest

The authors declare that they have no conflict of interest.

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