Review Article

Modernizing Thalassemia Detection: Insights from Third Generation Sequencing

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Abstract: Thalassemia, an inherited hematologic disorder, places a considerable societal and economic burden. Efficient prevention and management of this ailment necessitates the implementation of comprehensive screening strategies. Third–generation sequencing (TGS), an innovating technology, shows significant potential for applications in screening and diagnosis across numerous disorders. Although its utilization in the detection of thalassemia is in early phases, the current review explore recent and extensive applications, benefits of TGS, and associated problems and resolutions in incorporating it into routing screening and diagnostic procedures of thalassemia. In essence, TGS has displayed heightened degrees of progressive detection analysis and diagnostic precision in comparison to traditional methodologies and NGS. This observation suggests that technologies of TGS stands as a reasonable choice for diagnostic laboratories conducing the testing of thalassemia. The integration of TGS in diagnosis of thalassemia is anticipated to simplify the formulation of operational prevention and therapeutic approaches, ultimately mitigating impact of thalassemia on individuals and community.

Keywords: Thalassemia, Diagnosis, Third generation sequencing, Screening disorder, Full blood count, Red blood cell morphology.

INTRODUCTION

Thalassemia is cluster of hereditary hemolytic anemia disorders that arises from genetic alterations that result in absence/insufficiency to synthesize one or more chains of globins. In 1925, Dr. Thomas B. Cooley initially outlined the clinical manifestations of thalassemia [1]. Whipple and Bradford introduced phrase "thalassemia" for this anemic condition in 1936. The term is originates from Greek terms "thalassa", means "sea", and "haima", means "blood", emphasizing its prevalence among anemia individuals in Mediterranean regions [2]. The disease is also prevalent in the Southeast Asia, Middle East etc.

Thalassemia is categorized in two primary forms depending on involved chain of globin: α-thalassemia and β- thalassemia. On clinical background, it additionally segregates to Thalassemia major, severe form marked by profound anemia and related complications, Thalassemia minor, a milder variant with slight anemia and minimal clinical features, and Thalassemia intermedia, exhibiting a upper grade of anemia than minor but less dangerous as compared to major. Thalassemia major often necessitate regular blood transfusions, potentially leading to overload of iron and requiring chelation treatment to avert toxicity of iron [3].

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Hematopoietic Stem Cell Transplantations (HSCT) stands as possibly curative approach for the thalassemia, involving the infusion of normal hematopoietic stem cells [4]. Though, HSCT demands compatible donor and has inherent risks and clinical complications. Recent strides among gene therapy and editing, exemplified by the FDA – approved Zyntelgo and CRISPR gene – editing witth exagogene autotemcel, offer promising avenues [5]. However, steep cost of Zynteglo, prices at millions, has prompted concerns about accessibility.

Despite these advancements, thalassemia remains a substantial social and financial challenge, particularly in vastly endemic regions, with expected global occurrence of 5 to 7% carriers and yearly rate of birth exceeding 2.4%. In Pakistan, approximately 10 million carriers of thalassemia and nearly 5000 individuals having severe types contribute to escalating healthcare burden every year. The implementation of thalassemia screening programs is imperative to curtail new thalassemia cases and uphold targeted annual rate of birth for thalassemia major, ultimately diminishing lifelong costs of maintenance for the patients [6,7].

Traditional screening of thalassemia encompasses labor – intensive 3 – step process, including full blood count (FBC), assessment of red blood cell morphology, and hemoglobin (Hb) electrophoresis and/or high performance liquid chromatography (HPLC) [8]. Tests for confirmation, including the spanning break site assay (Gap-PCR), reverse spot hybridization assay (PCR-

RDB), multiplex linkage probe amplification (MLPA), or direct Sanger sequencing, are selected based on preliminary results. While considered the gold standard, these methods have limitations, particularly in precisely identifying uncommon mutations, necessitating evolution of novel screening tools for DNA [9]. Next-generation sequencing (NGS) surpasses electrophoresis in numerous ways, particularly in its ability to provide detailed and comprehensive genetic analysis. Electrophoresis is primarily a qualitative technique that separates DNA fragments based on size and charge, marking it useful for basic genetic screening or confirmation of specific markers [10]. However, it is limited in detecting complex genetic variations such as single nucleotide polymorphism (SNPs), insertions, deletions, or structural variations. NGS, on the other hand, provides a full spectrum of genetic information in a single run, offering both qualitative and quantitative insights. Unlike electrophoresis, which processes sample individually, NGS can handle multiple samples simultaneously, significantly increasing throughput and reducing analysis time for large-scale studies. Additionally, NGS produces digital data, which is more precise and reproducible than the band-based patterns observed in electrophoresis, minimizing human interpretation errors [10].

Next – generation sequencing (NGS), likewise called as second – generation sequencing (SGS), has gained widespread adoption but encounters limitations requiring supplementary techniques like Gap-PCR and Sanger sequencing [11]. Third – generation sequencing (TGS) emerges as a promising alternative, showcasing significant impending the detection of thalassemia [12]. Despite the high cost of NGS, many researchers and clinicians are adopting it because its advantages far outweigh its financial limitations. NGS provides unmatched sensitivity and specificity, enabling the identification of rare or low-frequency mutations that may be missed by traditional techniques. This makes it particularly valuable in diagnosing complex genetic conditions, such as thalassemia, where traditional methods like electrophoresis may fall short. Moreover, NGS consolidates multiple diagnostic steps into a single, streamlined process, eliminating the need for supplementary tests such as Sanger sequencing or Gap-PCR, which are often required after electrophoresis. The scalability and efficiency of NGS also contributes to its growing popularity. The ability to analyze hundreds or thousands of samples simultaneously makes it a preferred choice for large-scale genetic studies, and population screening programs. Another key advantage is the adaptability of NGS data. Once generated, the data can be stored and reanalyzed using newer algorithms or bioinformatics tools, extending its utility for future research and diagnostics. This adaptability ensures that NGS remains a forward-compatible and future-proof technology. While the initial cost is high, the long-term savings in terms of accuracy, speed, and diagnostic precision justify its adoption in clinical and research settings. The current review endeavors to explore modern applications, advantages problems, and resolutions related to incorporating TGS technology into conventional protocols for the diagnosis and screening of thalassemia.

THIRD GENERATION SEQUENCING

With development, third-generation sequencing (TGS), distinguished itself from traditional methods in direct sequencing of long DNA or RNA molecules. This technique observes the incorporation of nucleotides by DNA polymerase as it synthesize a single DNA molecule, producing long reads to 10 Kb with exceptional accuracy and low error rates [13]. In 2014, Oxford Nanopore Technologies (ONT) further advanced the TGS field by introducing nanopore sequencing. This breakthrough identifies variations as molecules of RNA or DNA traverse nanopore, introducing an innovative sequencing approach with long reads averaging tens of kilobases. Recent developments including additional TGS technologies like synthetic long – read sequencing (SLR) established by Illumina. SLR integrates long – range PCR and short – read sequencing to make long synthetic reads [14].

Technologies of TGS have transformed the research in genomics, enabling discovery of intricate genomic alterations that were once daunting or seemingly not possible to unravel. These advancements have broadened scope of genomic research by providing avenues for sequencing epigenomes, metagenomes, and long – read transcriptomes. Beyond realm of research, TGS has found practical application in clinical settings, playing a role in the diagnosis and management of various conditions [15-17].

THIRD GENERATION SEQUENCING IN THALASSEMIA

By a recent advancement in sequencing technologies, application of TGS for thalassemia has garnered significant attention. These investigations highlight the increasing recognition of TGS as a valuable asset in thalassemia screening and diagnosis (Table **1**).

Screening for Thalassemia Carrier Status at the Molecular Level

To mitigate occurrence of thalassemia major in newborns, it is crucial to enforce an obligatory strategy for mass screening along with suitable genetic counseling, particularly for populations at hazard. The utilization of TGS for thalassemia screening has recently come to the forefront, as described in Table **1**. An extensive and universally applicable framework for TGS screening, referred to as Comprehensive Analysis of Thalassemia Alleles (CATSA), was developed in 2021[15]. This technique entails a series of steps, encompassing extraction of DNA, PCR, and construction of library, sequencing, and analysis of bioinformatics. An essential aspect of the CATSA method involves employing an approach of multiplex PCR along with primers with long – range meticulously improved for specificity of the technique. This facilitates targeted amplicons generation covering all recognized regions of thalassemia, including deletions, insertions etc. With CATSA analysis, 1759 initial screening samples, identified through routine blood or hemoglobin (Hb) investigations, underwent screening. The outcomes underwent

validation through double – blind analysis and additional established procedures, showcasing a 100% accuracy rate for the test [15].

Numerous researches have been carried out to authenticate CATSA technique for screening of population, with the majority indicating enhanced rates of detection in comparison to standard techniques. For instance, a research study applied both CATSA and traditional methods to analyze patients suspected of having thalassemia. Notably, CATSA revealed 10 additional rare clinical variants, including α3.7III subtype, in comparison to the latter method [18]. These results imply that uncommon variants of thalassemia may be further widespread than assumed before and are frequently misdiagnosed using traditional diagnostic approaches. In a separate investigation, Liu *et al.* conducted investigations on individuals who had tested negative using particularly designed GAP-PCR and Sanger sequencing analyses [19]. CATSA recognized alterations in thalassemia gene in 8 individual, representing a greater rate of detection than conventional methods.

The carrier screening for potential parents has gained considerable consideration in numerous areas where compulsory premarital screening is applied, successfully alleviating thalassemia burden [20]. Recently, TGS is also employed in prenatal screening for thalassemia. In a study, couples were assessed for mutations of homozygous thalassemia utilizing TGS technology [21]. The results indicated that seven individuals were recognized as carriers, with five at risk of severe types in pregnancies, underscoring the effectiveness of TGS in screening parents in countries with a high prevalence of thalassemia.

Diagnosing Thalassemia through Molecular Methods

Evaluating the disease severity accurately is an essential phase in determining most suitable therapy, predominantly where patients manifest unusual symptoms or when traditional methods of genotyping provide undefined diagnosis. TGS, like NGS, provides the capability to directly identify thalassemia genotypes and interpret thalassemia variants efficiently. TGS has demonstrated success across numerous clinical circumstances, such as non – invasive prenatal diagnosis (NIPD), preimplantation genetic diagnosis (PGD), and diagnosing uncommon thalassemia cases [22].

Within the domain of PGF, Liu *et al.* assessed efficiency of TGS utilizing Oxford Nanopore sequencing platform, observing uniform analytical results when compared to traditional methods and NTGs [23]. Furthermore, budget of sequencing apparatus was found to be less as compared to NGS. Another study conducted by Wu *et al.* utilized TGS for examination of haplotype linkage, achieving successful haplotype linkage in 69% cases [24]. These findings aligned with those achieved through NGS, indicating encouraging efficiency for TGS in PGD. In research focused on NIPD, researchers assessed β – thalassemia among 13 families using TGS having relative haplotype dosage (RHD) analysis via Oxford Nanopore sequencing platform [25]. Their findings demonstrated effective haplotyping in each family when utilizing library of 20 kb amplicon, showcasing viability of TGS protocol. Advantage of TGS to detect as well as interpret genotypes of thalassemia emphasizes its ability to improve diagnostic correctness and facilitate modified treatment approaches for thalassemia patients.

TGS outperforms traditional detection techniques for thalassemia by identifying a vast array of α- deletion and β- deletion variants in singular analysis, marking significant leap over earlier detection capabilities. It also boosts the discovery rate of thalassemia genes, including those that are less common, by as much as 19%, thereby reducing the likelihood of missed diagnoses that can occur with older techniques. Furthermore, TGS increases diagnostic precision and reduces the chance of incorrectly diagnosing conditions, a common issue with traditional methods, particularly, in cases involving complex heterozygote. It facilitates straight identification of novel genetic variants of thalassemia and accurately distinguishes between cir-trans variations efficiently in single step, eliminating need for genetic confirmation from household members. Such advancement simplifies the analysis of thalassemia variants significantly. TGS also streamlines the testing process by doing away with requirement for numerous cross – validation and interpretation methods, therefore lowering the risk of contamination from complex steps [9,13].

In comparison to NGS, recent studies involving a large number of samples have revealed that TGS achieves a superior detection rate, especially in identifying deletion and heterozygotes [26, 27]. Such improvement is attributed to longer read lengths that TGS is capable of producing, which surpass those of NGS, thereby facilitating the easier identification of deletion variants in thalassemia genes. Healthcare professionals are increasingly favoring TGS above NGS for detection of long deletion mutations in thalassemia gene. Whereas, NGS struggles with sequencing areas that have unusual GC content and sequences that are highly repetitive, leading to overlooked mutations and false negatives, TGS overcomes these challenges with its longer read lengths and a more neutral approach to GC content. This results in more accurate and reliable rates of detection and diagnostic precision for thalassemic genes compared to NGS. An added benefit of TGS is the ability to deliver results more quickly than NGS, which often requires further validation through GapPCR and Sanger sequencing, thereby extending the duration of the testing process [22]. Various techniques are being used and are summarized in Table **2**.

Table 1. Efficacy of TGS in Detection of Thalassemia.

Application	Detection Rate	Outcome	Ref.
Screening	100%	Increased accuracy in genotype for individual carriers	[15]
	High	Identification of an additional 10 uncommon suboptimal mutations	[18]

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Screening	High	Discovery of extra mutations and one-step analysis of cis/trans conformational changes	[28]
	High	7% in uncommon thalassemia with identification of new three variants	[29]
	Same as TM	Equal detection rate in HKαα carriers, along with identification of two additional β-variants	[30]
	83.18%	Identification of uncommon genetic varations	[31]
	High	Highlighting that 5 couples had children with an increased risk of developing severe thalassemia	[21]
	Same as NGS	Rectification of NGS misdiagnosis in two heterozygotes	[26]
Genetic diagnosis (Preimplantation)	Same as NGS	Linkage analysis of haplotypes	[24]
	Same as NGS and TM	Uniform identification rates, but cost-effective	[23]
Genetic diagnosis (prenatal)	Same as NGS	Haplotyping in families with 20kb amplicon library	[25]
Diagnosis of rare variants	High	Identified additional uncommon genotypes and rectified the diagnosis of one heterozygote	[32]
	100%	Differentiation of various genotypes in a single step	[33]
	High	Detection of new deletion (10.3 kb) leading to α0-thalassemia	[27]
	Same as TM	Rectification of 3 misdiagnosed cases	[34]
	Same as TM	Capability to direct discern the structure of genotypes	[35]
	High	Diagnosis of 4 additional uncommon mutations associated with thalassemia	[36]
	High	Detection of new deletion (4.9 kb) with the β—globin gene	[37]
	High	Diagnosis of novel deletion (107 kb) within α-globin gene	[38]
	High	Identification of 8 additional uncommon mutations associated with thalassemia	[19]
	High	Detection of new deletion (7.2 kb) within the β-globin gene	[39]

NGS, next – generations sequencing; TGS, third – generation sequencing; TM, traditional method.

Table 2. Different New Techniques with Their Advantage and Disadvantages.

Technique	Advantage	Disadvantage
Third-Generation Sequencing (TGS)	- High sensitivity and specificity for detecting rare and complex mutations - Long-read capabilities improve accuracy for repetitive and GC-rich regions - Comprehensive one-step analysis eliminates supplementary tests. - Effective for identifying cis/trans conformation in a single analysis - Enhances diagnostic precision and reduces contamination risks	- High cost of equipment - Longer processing times for certain platforms - Requires expertise in library preparations and analysis
Nanopore Sequencing	- Real-time sequencing for rapid results - Portable and cost-effective - Supports both DNA and RNA sequencing	- Lower base-calling accuracy compared to HiFi reads - Requires advanced bioinformatics expertise

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Nanopore Sequencing	- No need for PCR amplification, preserving DNA integrity - Flexibility for field or clinical use	
Comprehensive Analysis of Thalassemia Alleles(CATSA)	- High accuracy for detecting thalassemia mutations - Identifies rare variants not detected by traditional methods - Multiple PCR with long-range primers ensures targeted specificity - Validates results through double-blind analysis	- Requires customized primers and computational tools - Limited to research and specific regions/ populations for validation
Synthetic Long-Read Sequencing (SLR)	- Integrates long-range PCR with short-read sequencing to reconstruct long reads - Effective for resolving repetitive and complex genomic regions - Provides insights into structural variations	- Additional steps increase complexity and cost - Limited availability compared to TGS or Nanopore technologies
High-Fidelity (HiFi) Reads	- Exceptional accuracy (99.99%) for detecting genetic variants - Resolves complex variations with high precision] - Effective for clinical and research applications, including parental and carrier screenings	- High equipment cost limits routine adaption - Slower processing time than Nanopore sequencing
Relative Haplotype Dosage Analysis (RHD)	- Facilitates non-invasive parental diagnosis (NIPD) for β-thalassemia	- Requires high-quality amplicon libraries
	- Successfully applied in high-risk pregnancies using TGS platforms - Reduces the need for invasive diagnostic procedures	
Single-Molecule Real-Time (SMRT) Sequencing	- Delivers highly accurate long reads - Effective for identifying rare variants and structural changes in thalassemia genes - Allows real-time sequencing of single DNA molecule	- High setup and maintenance costs - May require extended runtimes for complex analyses
Multiple-Ligation-Dependent Probe Amplification (MLPA)	- Affordable and effective for detecting deletions or duplications in thalassemia genes - Can complement sequencing approaches	- Limited resolution compared to NGS and TGS - Cannot detect single nucleotide variants (SNVs)
Gap-PCR	- Specific for identifying common deletion mutations in thalassemia - Cost-effective for targeted testing	- Limited to known deletion mutations - Cannot detect rare or novel variants
PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)	- Simple and affordable for identifying specific SNPs or mutations - Requires minimal equipment	- Low throughput and less precise compared to sequencing method - Cannot resolve complex genetic variations
Reverse Dot Blot Hybridization (RDB)	- Useful for detecting known mutations with high specificity - Straightforward protocol suitable for routine clinical testing	- Limited to known mutations - Lacks flexibility for novel variant detection

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CRISPR-based Diagnostics	- Emerging techniques with high specificity for detecting mutations - Potential for rapid point-of-care testing	- Currently limited to research settings

CRISPR, Clustered regularly interspaced short palindromic repeats; PCR, polymerase chain reaction.

CHALLENGES

The precision of TGS in diagnosing diseases has been under scrutiny and subject to enhancement for an extended period, which has cast some doubt on its diagnostic utility. Yet, recent improvements has revealed TGS's sequencing accuracy to a level on par with other published sequencing methods, such as those by Illumina sequencing. Early iterations of the PacBio sequencing platform were known for their substantial error rates, approximately 13%, but the latest technology, particularly the high – fidelity (HiFi) reads from Circular Consensus Sequencing (CCS), boasts impressive correctness rate of 99.99% [40,41]. In a smaller vein, the initial error rates of ONT technology ranged from 5% to 25%, but the newest version 10 flow cells claim to achieve base – calling accuracy of up to 99.99% [42]. By integrating unique molecular identifiers (UMIs) along with either PacBio CCS or ONT in an amplicon sequencing strategy, the accuracy can surpass 99.99% [43]. Thus, accurateness of TGS is now not an impediment to the reliable detection of thalassemia.

The PacBio platform is the leading choice for detection of thalassemia through TGS. Despite its advanced technical capabilities, the substantial cost of PacBio's equipment and the extended duration required for processing are barriers to its routine use in screening and diagnostics. To overcome the challenges of high equipment cost.

CONCLUSION

As a cutting – edge approach, third - generation sequencing (TGS) is still gaining traction for use in thalassemia screening and diagnosis on a global scale. Recent evaluations and exploratory researches have indicated that TGS, especially with PacBio system, significantly outperforms traditional methods and next – generation sequencing (NGS) in detecting thalassemia with higher accuracy. However, the widespread adoption of PacBio for TGS in regular thalassemia testing is hindered by high cost of equipment and the time it takes to obtain results. The ONT platform, which is more cost – effective and yields faster outcomes, shows promise for TGS in thalassemia genetic testing, but its full potential is yet to be realized. Extensive trials are necessary to establish its effectiveness and precision.

AUTHORS' CONTRIBUTION

Shahzad Ali Jiskani: Conceptualization, Writing draft, Critical review and revision the manuscript.

Asma Mustafa, Maryam Zulfiqar, Bushra Anam Ali, Aliena Sohail and **Asfa Zawar**: Methodology, data analysis and interpretation, Writing draft, Critical review and revision the manuscript.

CONFLICT OF INTEREST

Declared none.

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