Advanced Methods for Detection of Haemoglobinopathies

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ABSTRACT

Haemoglobinopathies, a group of genetic disorders affecting the structure or production of haemoglobin, remain a significant public health concern globally, particularly in regions such as sub-Saharan Africa, Southeast Asia, and the Mediterranean. These disorders include conditions like sickle cell disease and various forms of thalassemia, which necessitate early and accurate diagnosis for effective management and treatment. Normal adult haemoglobin (HbA) is designated $\alpha^{A}{}_{2}\beta^{A}{}_{2}$. Variant haemoglobin is derived from gene abnormalities affecting the α-globin genes (HBA1 or HBA2) or β-globin (HBB) structural genes. More than a thousand haemoglobin variants have been identified relative to changes in the globin chains. Advanced methods for the detection of haemoglobinopathies have dramatically improved our diagnostic capabilities, facilitating more precise and comprehensive investigation of these conditions. The use of next-generation sequencing (NGS), permits the screening of multiple genes linked with haemoglobinopathies, Polymerase chain reaction (PCR)-based techniques, such as quantitative PCR (qPCR) and droplet digital PCR (ddPCR) are essential tools for investigating specific mutations in haemoglobin genes, the integration of CRISPR-Cas9 technology for gene editing provides a promising stage for both diagnostic and therapeutic applications in haemoglobinopathies, while Advancements in mass spectrometry have also expressively facilitated to the detections of haemoglobinopathy. These would definitely help in the development of targeted therapeutic strategies and personalized management plans. Data were generated from PubMed, Google Scholar, Nature, BMC, Tailor and Francis, MDPI, Springer, and some other related data. This review was aimed to provide appreciable information on the current methods for the investigation of haemoglobinopathies.

Keywords: Haemoglobinopathies, Haemoglobin Variants, Advanced Methods

INTRODUCTION

Haemoglobin is a tetramer composed of two α–globin and two non–α– globin chains (protein) groups attached to the haem (prosthetic) group to transport oxygen and carbon dioxide within the blood (Bunn, *et al.,* 2019). The distribution of haemoglobin genotypes varies from one population to another. Nigeria is one of the African Countries that are vulnerable to hereditary erythrocyte disorders such as sickle cell disease, haemolytic disease of the new born and haemoglobinopathies (Nnodu, *et al.,* 2019). Haemoglobin been one of the most essential component of red blood cells have multiple functions for the wellbeing of human. It includes transport of oxygen from the lungs to the tissues mostly to facilitate oxidative phosphorylation in the mitochondria, carriage of carbon dioxide from tissues to the lungs, buffering of hydrogen ions formed in the erythrocyte from the conversion of carbon dioxide into bicarbonate (Power *et al.,* 2018). In humans, six main types of haemoglobins are present, and the different types are seen in different stages of human development. The normal haemoglobin is as follows based on the different stages of development, from embryonic to foetal to adult types: Hb Gower-1 ($\delta^2\varepsilon^2$), Hb Gower-2 ($\alpha^2\varepsilon^2$), Hb Portland -1 ($\delta^2\gamma^2$), Hb Portland -2 ($\delta^2\beta^2$), Foetal Hb ($\alpha^2\gamma^2$), HbA2 ($\alpha^2\delta^2$) and HbA ($\alpha^2\beta^2$) (Weatherall, *et al.,* 2020; Bunn, *et al.,* 2019). Other forms of haemoglobin exist. Examples are haemoglobin S (HbS) and haemoglobin C (HbC) (Bunn *et al.,*

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2019). Traditional diagnostic methods, such as haemoglobin electrophoresis and high-performance liquid chromatography (HPLC), continue to be valuable for identifying haemoglobin variants. However, innovations in molecular diagnostics have revolutionized haemoglobinopathy testing by offering enhanced sensitivity, specificity, and speed (Mei, *et al.*, 2020).

Recent advancements in investigation methods have dramatically improved our diagnostic capabilities, enabling more precise and comprehensive analysis of these disorders (Chan, *et al.*, 2018). One such advancement is the use of next-generation sequencing (NGS), allows for the simultaneous screening of multiple genes associated with haemoglobinopathies, providing a comprehensive genetic profile at a much faster rate and lower cost compared to earlier methods (Weatherall, 2016). Polymerase chain reaction (PCR)-based techniques, including quantitative PCR (qPCR) and droplet digital PCR (ddPCR), have become essential tools for detecting specific mutations in haemoglobin genes (Hoban, *et al.*, 2016). These methods facilitate the identification of carriers and affected individuals with high precision, enabling better genetic counselling and risk assessment (Chan, *et al.*, 2018). Furthermore, the integration of CRISPR-Cas9 technology for gene editing holds promise for both diagnostic and therapeutic applications in haemoglobinopathies (Hoban, *et al.*, 2016). Advancements in mass spectrometry have also significantly contributed to haemoglobinopathy diagnostics. Mass spectrometry-based techniques offer precise quantification and characterization of haemoglobin variants, enhancing the accuracy and reliability of diagnostic testing (Mei, *et al.*, 2020). These recent investigation methods not only improve diagnostic accuracy but also provide insights into the pathophysiology of haemoglobinopathies. This review explored the specifics of these advanced techniques, highlighting their impact on the field of haemoglobinopathy diagnostics.

HAEMOGLOBIN AND HAEMOGLOBIN SYNTHESIS

Haemoglobin

Haemoglobin is a protein containing iron that facilitates the transport of oxygen in red blood cells. Haemoglobin in the blood carries oxygen from the respiratory organs to the other tissues of the body, where it releases the oxygen to enable respiration. A healthy human has 12 to 20 grams of haemoglobin in every 100 mL of blood.

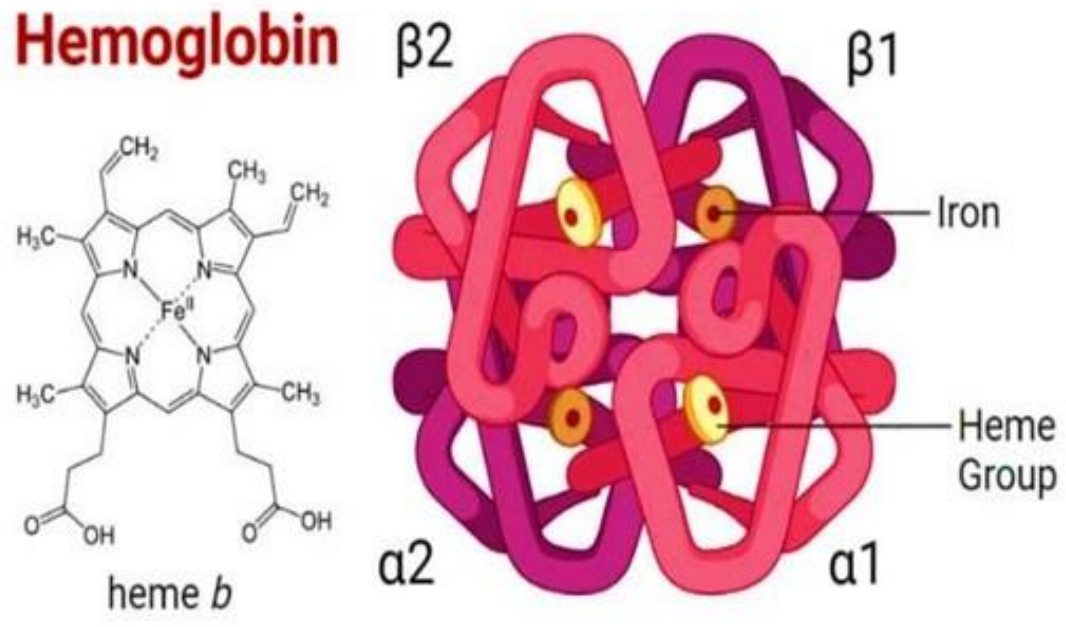

Figure 1: Haemoglobin Molecule. α; alpha globulin, β; beta globulin (Modified from Dahal, 2023).

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Each haemoglobin molecule is made up of four heme groups surrounding a globin group, forming a tetrahedral structure (Fig. 1 & 2) (Ponka *et al.,* 2020). The heme, which makes up only 4% of the molecule's weight, is composed of a ring-like organic compound known as a porphyrin to which an iron atom is attached (Kaplan and Hoyer, 2022). It is the iron atom that binds oxygen as the blood travels between the lungs and the tissues. There are four iron atoms in each molecule of haemoglobin, which accordingly can bind four molecules of oxygen (Leder *et al.,* 2022). Globin consists of two linked pairs of polypeptide chains, These four polypeptide chains (2 alpha and 2 beta chains) are arranged in a specific quaternary structure to form the globin molecule (Kazazian *et al.,* 2020). The globin molecule then combines with four heme groups, each containing an iron atom, to form the complete haemoglobin molecule (Ponka *et al.,* 2020).

Biochemical Synthesis of Haemoglobin

Haemoglobin (Hb) is a tetrameric molecule made up of 2 alpha-like (ζ or α) and 2 beta-like globin chains (ε, γ, δ or β), with each containing a haem group attached which serves as an oxygen carrier protein in the red cells. There are approximately 250 million haemoglobins in one erythrocyte (Kwaifa *et al*., 2020). The two main components of haemoglobin synthesis are globin production and haem synthesis. Haem synthesis occurs in both the cytosol and mitochondria of erythrocytes. It begins with lysine and succinic coenzyme A and ends with the production of protoporphyrin to Fe^{2+} ion, forming the final haem molecule (Ponka *et al.*, 2020). Human blood has a few different forms of normal haemoglobin. The percentage prevalence of haemoglobin depends on the stage of development. During pregnancy, the foetus primarily produces foetal haemoglobin (HbF). HbF comprises two α- and two γ-globin (α_2 γ_2) subunits. HbF has a stronger oxygen affinity than HbA, allowing oxygen to flow from maternal oxygen affinity than HbA, allowing oxygen to flow from maternal to foetal circulation through the placenta. Production of HbF starts at around week 6 of pregnancy and drops significantly after birth, reaches low, near-adult levels by two years, and ultimately makes up 2 to 3% of haemoglobin in adults. HbA, the most common adult form of haemoglobin, comprises two alpha and two beta-globin subunits. Inversely to HbF, HbA production explodes after birth and ultimately makes up 95-98% of adult haemoglobin. HbA2 is a less common adult form of haemoglobin. It comprises two alpha and two delta-globin sub-units, making up 1 to 3% of haemoglobin in adults (Ponka *et al.,* 2020).

There are multiple steps involved in haem synthesis. Eight enzymes accomplish this process, four working in the mitochondria and four in the cytosol. The process starts in the mitochondria, where ALA (aminolevulinic acid) synthase links glycine and succinyl coenzyme A to form ALA. Steps 2 through 5 occur in the cytosol. Next, ALA dehydratase takes two molecules of ALA and produces porphobilinogen (PBG). In the third step, porphobilinogen deaminase takes four molecules of PBG and produces hydroxymethylbilane. Next, uroporphyrinogen III cosynthase takes hydroxymethylbilane and produces uroporphyrinogen III. In the fifth step, uroporphyrinogen decarboxylase takes uroporphyrinogen III and produces coproporphyrinogen III. The final three steps of haem synthesis occur in the mitochondria. Coproporphyrinogen III is then transformed into protoporphyrinogen IX by coproporphyrinogen oxidase. The seventh step occurs when protoporphyrinogen oxidase converts protoporphyrinogen IX to protoporphyrin IX. The eighth and final step of haem synthesis is the addition of Fe to protoporphyrin IX by ferrochelatase, producing a haem molecule. In haemoglobin synthesis, errors can arise in numerous steps and processes. For example, haem synthesis involves multiple enzymes. Issues arise when one of these enzymes is deficient or inadequately functioning. During globin sub-unit production, potentially serious consequences can occur if there are mutations or deletions in genes coding for globin chains; this relates to disorders in which abnormal haemoglobin predominates in the blood (haemoglobinopathies) (Kwaifa *et al*., 2020).

Globin chain production occurs at the erythrocyte cytosol and by transcription and translation. Many studies have shown that the presence of haem induces globin gene transcription. The alpha-globin gene cluster is situated at the short arm of chromosome 16 (16p13.3) (as 5′-ζ-μ-α_2-α1–3′). Clusters of the alpha globin genes are arranged according to the order in which they are expressed during the developmental period. On the

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI | Volume XI Issue XV November 2024 | Special Issue on Public Health

other hand, the beta-globin gene locus is located on chromosome 11 (11p15.5) as 5′-ε-Gγ-Aγ-δ-β-3'. Hb Gower-I ($\zeta_2\varepsilon_2$), Hb Gower-II ($\alpha_2\varepsilon_2$), and Hb Portland ($\zeta_2\gamma_2$) are synthesized at early embryonic life, and foetal haemoglobin (HbF, $\alpha_2\gamma_2$), which predominates throughout the foetal life. Postnatally, the foetal haemoglobin switches to HbA_2 (HbA_2, $\alpha_2\delta_2$) and HbA ($\alpha_2\beta_2$) (96–98%) (Fig. 3). Alpha-globin genes are expressed consistently at high levels from the beginning of foetal development. Hence, the effects of α-globin gene mutations are manifested throughout the foetal and adult life. This is quite different from the mutations of β-globin genes which exert their effects only approximately 6 month after birth. Generally, the collective production of alpha-globin chains from the four alpha-globin genes on chromosome 16 is estimated to be equalled to the total synthesis of the beta-globin chains derived from the two beta-globin genes on chromosome 11 (Kwaifa *et al.*, 2020).

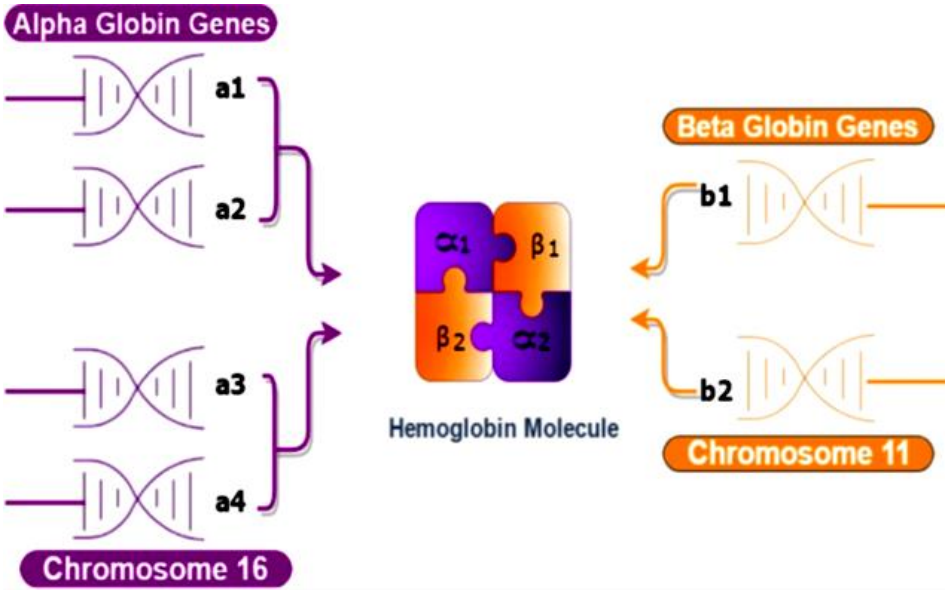

Figure 2: Globin genes in normal haemoglobin molecule. a; alpha globulin, b; beta globulin (Modified from Kwaifa *et al.*, 2020)

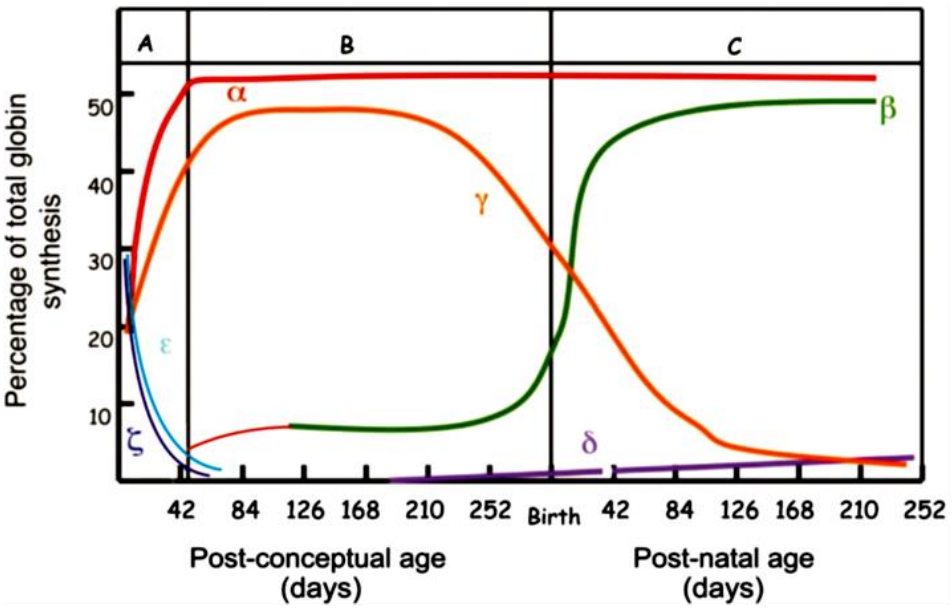

Figure 3: Haemoglobin synthesis during developmental stages.

The age of the foetus/baby in days is represented in the x-axis while the y-axis indicates the percentage of the total globin genes expressed. The vertical lines indicate the time of birth. Within the first 42 gestational days, the embryonic genes and the first switching from ε- to γ-globin genes take place. The second switching

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of γ- to β-globin takes place soon after birth. α; alpha globulin, β; beta globulin (Modified From Kwaifa *et al.,* 2020)

HAEMOGLOBINOPATHIES

Haemoglobinopathies are genetic disorders affecting the structure or production of haemoglobin, the protein in red blood cells responsible for oxygen transport. These disorders are typically inherited in an autosomal recessive pattern. The two main types of haemoglobinopathies are structural haemoglobin variants and thalassemias (Higgs *et al.,* 2019).

Haemoglobin Variants

Haemoglobin variants arise from mutations in the globin genes, leading to altered haemoglobin molecules (Piel *et al.,* 2017). The inherited disorders of haemoglobin are the most common genetic disorder worldwide, with 7% of the world's population being carriers. It is on record that about 300.000 children are born with sickle cell disease (SCD) worldwide every year. The most common types of haemoglobinopathies are the sickle cell disorder and the thalassaemias, occurring in people of African, Asian, South European and Middle Eastern descent. These sickling disorders include the heterozygous state for haemoglobin (HbAS), the homozygous for haemoglobins (HbSS) and the compound heterozygous state for haemoglobins together with haemoglobin C, D, E or other structural variants (Kwaifa *et al*., 2017). The most well-known example is sickle cell disease (SCD), caused by a mutation in the β-globin gene. This mutation results in the production of haemoglobin S (HbS), which can polymerize under low oxygen conditions, causing red blood cells to assume a sickle shape. These sickled cells can block blood vessels, leading to pain, organ damage, and increased risk of infection (Piel *et al.,* 2017).

Thalassemias

Thalassemias are characterized by reduced or absent production of one or more of the globin chains that make up haemoglobin. This imbalance leads to ineffective erythropoiesis and haemolysis. The two main types are alpha thalassemia and beta thalassemia, depending on whether the α-globin or β-globin chain production is affected (Higgs *et al.,* 2019).

Alpha Thalassemia

Alpha thalassemia results from deletions or mutations in one or more of the four α-globin genes (HbA_1 and HbA_2). The severity of the disease depends on the number of affected genes. Individuals with one or two gene deletions are often asymptomatic or have mild anaemia, while those with three deletions have haemoglobin H disease, which can cause moderate to severe anaemia. The deletion of all four genes results in haemoglobin Bart's hydrops foetalis, a condition that is usually fatal before or shortly after birth (Higgs *et al.,* 2019).

Beta Thalassemia

Beta thalassemia is caused by mutations in the β-globin gene, leading to reduced (β+) or absent (β0) β-globin synthesis. The severity of beta thalassemia varies based on the nature of the mutation and whether one or both β-globin genes are affected. Beta thalassemia major (Cooley's anaemia) is the most severe form, requiring regular blood transfusions and iron chelation therapy to manage complications such as iron overload (Taher *et al.,* 2018).

Emerging Treatments

Recent advances in gene therapy and genome editing offer promising new approaches to treating haemoglobinopathies. Techniques such as CRISPR-Cas9 are being explored to correct the genetic mutations responsible for these disorders, potentially providing a cure (Orkin and Bauer, 2019).

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI | Volume XI Issue XV November 2024 | Special Issue on Public Health

TRADITIONAL METHODS FOR THE INVESTIGATIONS OF HEMOGLOBINOPATHIES

Traditional diagnostic methods for these conditions have been crucial in early detection and management. These include:

Complete Blood Count (CBC)

The routine analysis to diagnose a haemoglobinopathy carrier involves a complete red blood count including Haemoglobin concentration (Hb g/l), Mean Cellular Volume (MCV fl), Mean Corpuscular Haemoglobin (MCH pg), Packed Cell Volume (PCV l/l), and Red Blood Cell count (RBC × 1012 /L). Instruments for analysing most of these parameters can be automated, such as automated electronic cell counters. In addition, haptoglobin (Hp) and Zinc Protoporphyrin (ZPP) or ferritin are markers used in relationship to the complete red blood cell count (i.e., low Hb, RBC and PCV) to distinguish haemolysis or iron deficiency anaemia (low Hb, MCV, MCH and low/normal RBC and elevated ZPP or low ferritin/SeFe). If the MCV and MCH are low at normal iron levels, indicating the microcytic hypochromia, a presumptive diagnosis of a thalassemia carrier can be made. Hb variants, such as the common HbS, HbC or HbD variants for example may present with normal parameters and remain undetected by hematologic analysis (Dwivedi *et al.,* 2024; Harteveld *et al.,* 2022; Agnello *et al.,* 2021).

Peripheral Blood Smear

A peripheral blood smear involves microscopic examination of a blood sample. It helps in identifying morphological abnormalities in RBCs. For instance, sickle cells, target cells, and Heinz bodies can indicate specific haemoglobinopathies (Fig. 4) (Rachmilewitz and Giardina, 2020).

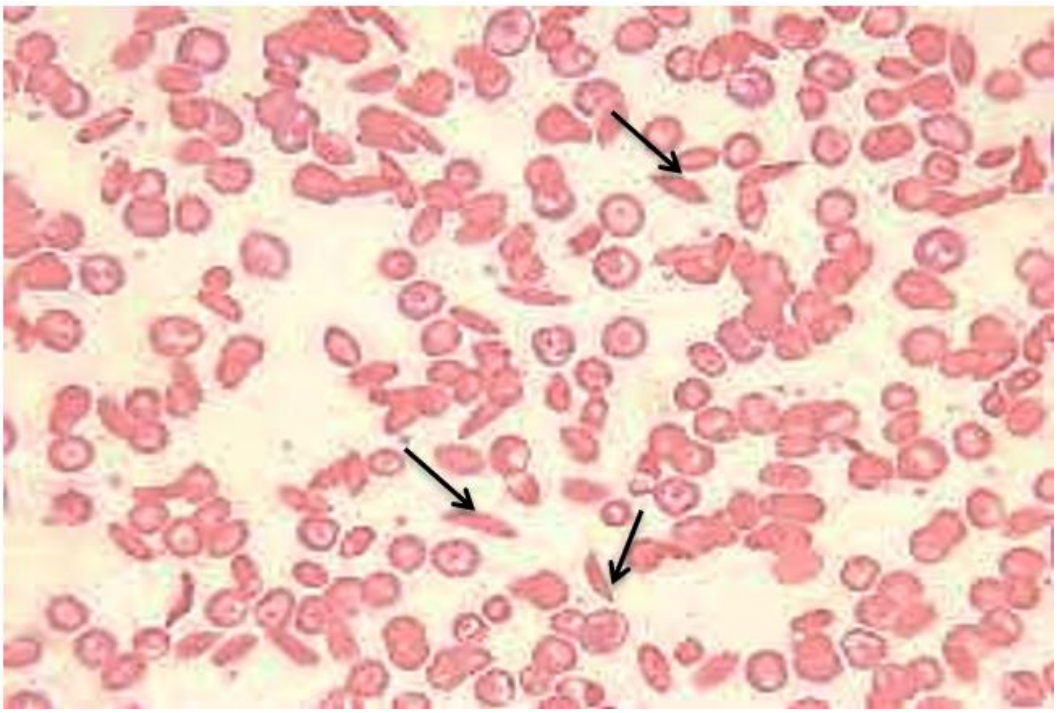

Figure 4: Peripheral blood smear indicating sickled cells (Black arrow) (Uthman, 2024).

Haemoglobin Electrophoresis

Haemoglobin electrophoresis is a key diagnostic test that separates different types of haemoglobin based on their electrical charge. It can identify abnormal haemoglobin variants, such as HbS in sickle cell disease and HbF and HbA_2 in thalassemia (Fig. 5) (Weatherall and Clegg, 2018).

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI | Volume XI Issue XV November 2024 | Special Issue on Public Health

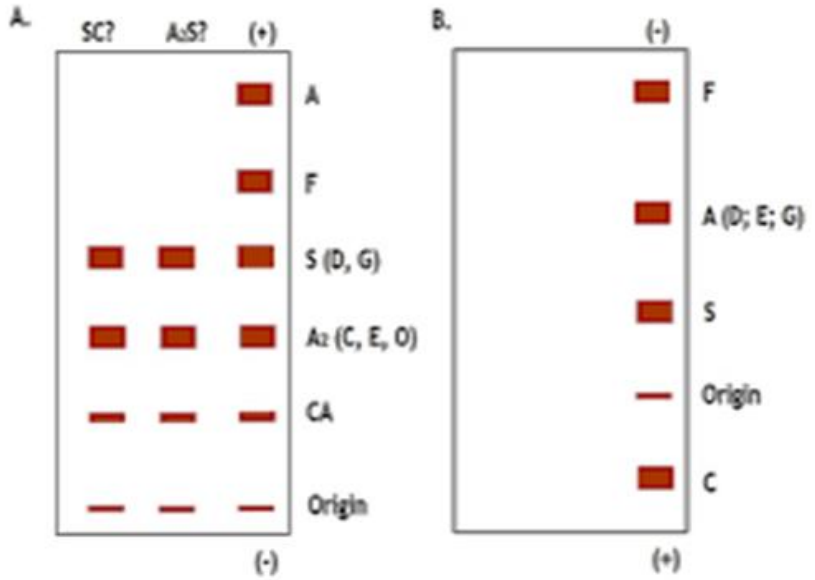

Figure 5: Migration of haemoglobin on a cellulose acetate paper (Weatherall and Clegg, 2018; Adu et al., 2017).

Sickling Test

The Sickling test is a traditional method used to detect sickle haemoglobin (HbS) in red blood cells. The test is based on the principle that HbS causes red blood cells to sickle or distort when exposed to low oxygen levels. The Sickling Test has largely been replaced by more modern and accurate techniques, such as HPLC and DNA analysis, but it may still be used in some settings as a preliminary screening test (Weatherall and Clegg, 2018).

Solubility Test

The Solubility test, also known as the Sickling test or Haemoglobin Solubility Test, is a traditional method used to detect the presence of sickle haemoglobin (HbS) in red blood cell. The test is based on the principle that HbS is less soluble in a reducing solution than other haemoglobin variants (Bunn *et al.*, 2020).

LIMITATIONS OF TRADITIONAL METHODS FOR THE INVESTIGATIONS OF HAEMOGLOBINOPATHIES

While traditional methods for detecting haemoglobinopathies have been valuable, their limitations highlight the need for more advanced techniques. Molecular methods and next generation sequencing offer higher sensitivity, specificity, and ability to detect a wider range of haemoglobin variants, providing more comprehensive diagnostic information (Jones *et al.*, 2020).

Low Sensitivity and Specificity

Traditional methods such as haemoglobin electrophoresis, High-Performance Liquid Chromatography (HPLC), and solubility tests often lack the sensitivity and specificity required to accurately diagnose all haemoglobinopathies. These methods may fail to detect minor or rare variants of haemoglobin. For example, haemoglobin electrophoresis can sometimes misinterpret or overlook small but clinically significant haemoglobin variants (Jones *et al.*, 2020).

Inability to Detect Silent Mutations and requires expertise in interpretation.

Many traditional methods focus on identifying abnormal haemoglobin based on its electrophoretic mobility or solubility properties. However, they may miss silent mutations that do not affect these properties but still

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI | Volume XI Issue XV November 2024 | Special Issue on Public Health

cause clinical symptoms. These silent mutations often require molecular methods for detection, which are not part of traditional screening approaches (Smith and Brown, 2019).

Time-Consuming and Labour-Intensive

Techniques like haemoglobin electrophoresis and HPLC are labour-intensive and time-consuming, requiring significant manual intervention and interpretation by skilled personnel. This can limit their utility in high-throughput settings and make them less suitable for large-scale population screening (Doe *et al.,* 2018).

Limited Scope of Detection

Traditional methods are generally designed to detect specific, well-known haemoglobin variants such as HbS, HbC, and HbE. However, there are over 1,000 known haemoglobin variants, and traditional methods are not equipped to identify many of these. As a result, patients with rare or newly discovered haemoglobinopathies may go undiagnosed (Green *et al.,* 2021).

Interference and Misdiagnosis

Certain factors can interfere with traditional testing methods, leading to misdiagnosis. For example, the presence of high levels of foetal haemoglobin (HbF) can interfere with haemoglobin electrophoresis results. Additionally, conditions such as iron deficiency anaemia can alter haemoglobin levels and lead to inaccurate interpretations (White *et al.*, 2022).

Lack of Genetic Information

Traditional methods do not provide genetic information about the mutations responsible for the haemoglobinopathy. Understanding the specific genetic mutations involved is crucial for accurate diagnosis, prognosis, and family planning. Molecular genetic techniques, such as DNA sequencing, are required to obtain this information but are not included in traditional haemoglobinopathy screening protocols (Brown *et al.*, 2020).

ADVANCED METHODS IN DETECTING HEMOGLOBININOPATHIES

Recent advances in molecular and genetic techniques have significantly enhanced the accuracy and efficiency of haemoglobinopathies detection.

DNA-Based Techniques in the Detection of Haemoglobinopathies

DNA-based techniques have significantly improved the detection of these disorders, offering precise and reliable results. DNA-based techniques include:

CRISPR- Based Detection Technology

CRISPR technology has revolutionized genetic engineering and has shown promise in detecting genetic disorders for changing in gene function and DNA sequences (Fig. 6). CRISPR uses a guide RNA to direct the Cas protein to a complementary target sequence, resulting in a detectable signal. This method can also be programmed to detect specific DNA sequences associated with genetic disorders, such as mutations, deletions, or insertions in disease-causing genes. CRISPR-Cas systems can also be adapted to detect RNA sequences, enabling the detection of RNA viruses or transcripts associated with genetic disorders (Ansori *et al.*, 2021). CRISPR-based methods can also detect single nucleotide polymorphisms (SNPs) associated with genetic disorders. Multiplexed detection allows for efficient and cost-effective screening for a panel of genetic disorders. CRISPR-based nucleic acid detection methods can be adapted for point-of-care testing, enabling rapid and decentralized diagnosis of genetic disorders. These methods offer high sensitivity and specificity, allowing for the detection of low abundance genetic variants associated with genetic disorders.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI | Volume XI Issue XV November 2024 | Special Issue on Public Health

CRISPR technology is also being used to study the underlying genetic mechanisms of various disorders, providing insights into disease pathology and developing novel therapeutic strategies. It also applied in genetic abnormalities modifications, and prevention of disease transmission (Dwivedi *et al.,* 2024; Shademan *et al.,* 2022).

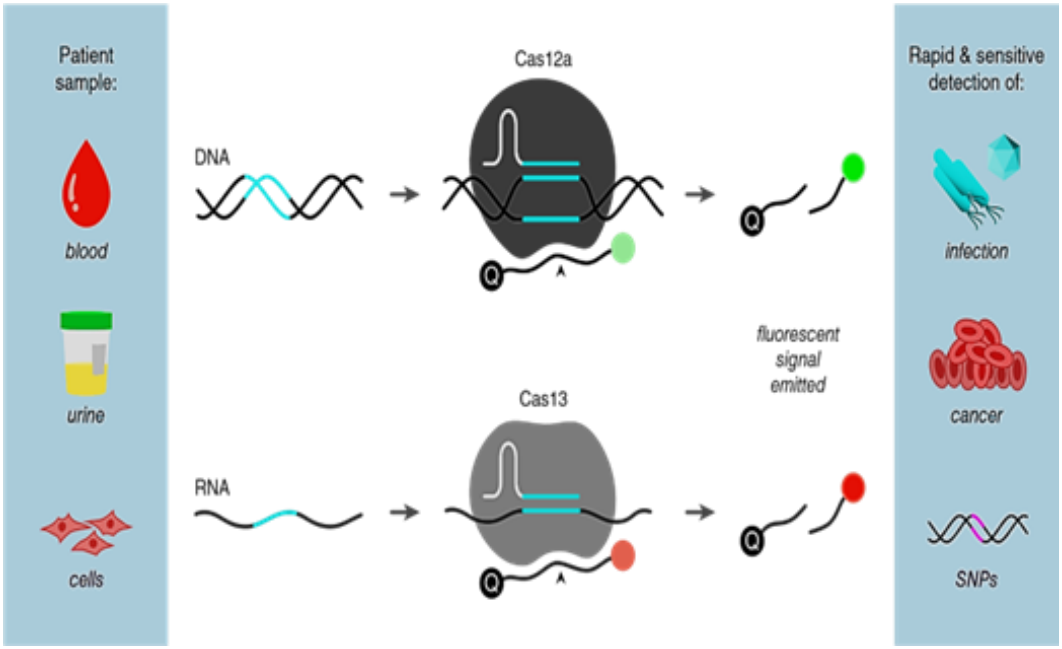

Figure 6: Diagnostic applications of CRISPR-Cas enzymes.

The ability to recognize precise genetic sequences allows use of CRISPR-Cas enzymes for biomedical testing. Cas12a (top) and Cas13 (bottom) can respectively be programmed to recognize DNA or RNA, activating the enzymes to cleave nearby nucleic acid molecules. Single-stranded DNA or RNA molecules bearing a fluorophore and a quencher (Q) are provided as part of the detection system, and these will result in a fluorescent signal following cleavage by Cas12a or Cas13, respectively. Because this signal will only result if the CRISPR-Cas enzyme finds a sequence matching its targeting RNA (cyan), this system can perform exquisitely sensitive detection of genetic sequences associated with pathogens or disease (Modified from Foss *et al*., 2018).

Polymerase Chain Reaction (PCR): PCR is a widely used technique to amplify specific DNA sequences, allowing for the detection of point mutations, deletions, and insertions in haemoglobin genes.

Allele-Specific PCR (AS-PCR): This method targets specific mutations, such as those found in sickle cell disease. It uses primers designed to bind to the mutant allele, enabling selective amplification and detection (Tahar, *et al.,* 2018).

Multiplex PCR: This variant allows simultaneous amplification of multiple DNA targets, making it suitable for detecting various thalassemia mutations in a single reaction (Harteveld *et al.,* 2023; Yadav, *et al.,* 2017).

Amplification refractory mutation system PCR (ARM- PCR): In genetic disease diagnostics, ARM-PCR is one of the most convenient methods now a day. PCR is a powerful tool for detecting diseases by identifying specific regions of the pathogen's genome. Amplification refractory mutation system PCR is able to detect single base substitutions such as in haemoglobinopathies. This has led to improved specificity because allele specific primers will amplify DNA only in the presence of the allele. In the single-ARMS-PCR each SNP is analysed by the use of three primers which include a common primer and two allele specific primers. The objective was to produce a protocol that is simple, less expensive and less time consuming, and that can be widely applicable in resource limited settings. (Dwivedi *et al.,* 2024; Harteveld *et al.,* 2023)

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI | Volume XI Issue XV November 2024 | Special Issue on Public Health

Reverse Dot Blot Hybridization: Reverse dot blot hybridization involves hybridizing PCR-amplified DNA to specific probes immobilized on a membrane. It is useful for detecting known mutations in haemoglobinopathies. This technique is employed in thalassemia screening programs to identify carriers of common mutations (Salem-Bekhit *et al.,* 2019).

Next-Generation Sequencing (NGS): NGS has emerged as a powerful tool for comprehensive genetic analysis, allowing the sequencing of entire genes or genomes to provide detailed information on both known and novel mutations (Dwivedi *et al.,* 2024).

Whole Exome Sequencing (WES): WES focuses on coding regions of the genome, where most disease-causing mutations occur. It is effective in identifying rare and complex haemoglobinopathy mutations (SeyedAhmad, *et al.,* 2020).

Targeted Gene Panels: These panels sequence specific genes associated with haemoglobinopathies, offering a balance between depth of coverage and cost (Raja *et al.,* 2022).

High-Performance Liquid Chromatography (HPLC)

HPLC is another crucial method used to identify and quantify different haemoglobin fractions. It offers higher resolution and sensitivity compared to electrophoresis. HPLC is particularly useful for newborn screening and diagnosing carriers of haemoglobinopathies (Arzanlou *et al.,* 2021). HPLC separates haemoglobin variants based on their charge, size, and hydrophobicity, allowing for sensitive and specific detection (Kazazian *et al.,* 2020).

Mass Spectrometry (MS)

Mass spectrometry (MS) has emerged as a powerful tool for the detailed analysis of haemoglobin variants, offering high sensitivity, specificity, and the ability to detect multiple variants simultaneously (Nadarajan *et al.,* 2018). Mass spectrometry (MS) is a powerful analytical technique used to detect and identify hemoglobinopathies (Kleanthous *et al.,* 2019). MS measures the mass-to-charge ratio of ions, allowing for the identification of haemoglobin variants based on their molecular weight (Wild *et al.,* 2020).

Principle: The technique utilizes electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) to ionize haemoglobin molecules, which are then separated and detected based on their mass-to-charge ratio (Daniels *et al.,* 2018).

Types of Mass spectrometry

1. Electrospray Ionization Mass Spectrometry (ESI-MS)
2. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)
3. Tandem Mass Spectrometry (MS/MS).

Isoelectric Focusing (IEF)

Isoelectric focusing separates proteins in a gel medium with a pH gradient consisting of ampholytes (zwitterions). When a high voltage is applied, narrow buffered zones are created with stable but slightly different pH. Slower-moving proteins migrate through these zones and stop at their isoelectric points (pI). In the case of haemoglobin, these migrate to a zone in the medium where the pH of the gel matches the haemoglobin pI. At the pI, the charge of the haemoglobin becomes zero and ceases to migrate. The haemoglobin migration order of IEF is similar to alkaline electrophoresis. Resolution is clear with the differentiation of HbC from HbE and HbO and HbS from HbD and HbG, respectively. HbA and HbF are also clearly differentiated. A distinct band representing acetylated HbF is readily identified with neonatal specimens and slightly anodic to HbA. Staining may be necessary depending on the manufacturer's recommendations. Gels may be read wet or dry, and band identification is accomplished compared to

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI | Volume XI Issue XV November 2024 | Special Issue on Public Health

migration patterns of known quality controls. This method allows for greater precision and accurate quantification than standard electrophoresis. It gives excellent resolution in addition to high throughput but is labour-intensive, and time-consuming. An alternative method is needed to confirm or differentiate haemoglobin variants (bands other than HbF or HbA). IEF can identify fast migrating bands (Hb Bart's, HbH) (Weatherall and Clegg, 2018).

ADVANTAGES OF ADVANCED METHODS FOR THE INVESTIGATIONS OF HAEMOGLOBINOPATHIES

Recent advancements in diagnostic methods for detecting haemoglobinopathies have significantly enhanced their accuracy, speed, and comprehensiveness. Key methodologies include next-generation sequencing (NGS), high-performance liquid chromatography (HPLC), and mass spectrometry (MS), among others (Guo *et al.,* 2020).

Next-Generation Sequencing (NGS)

Advantages:

1. High Throughput and Sensitivity: NGS can analyse multiple genes simultaneously, allowing for the detection of a wide range of mutations associated with haemoglobinopathies in a single run (Guo *et al.,* 2020).
2. Comprehensive Genotyping: It can identify rare and novel variants that traditional methods might miss, providing a comprehensive genetic profile of patients (Wong *et al.,* 2021).
3. Accuracy: NGS has a high accuracy rate for detecting both known and novel mutations, reducing the risk of misdiagnosis (Gimelli *et al.,* 2022).

High-Performance Liquid Chromatography (HPLC)

Advantages:

1. Precision and Reproducibility: HPLC provides precise quantification of different haemoglobin variants, which is crucial for accurate diagnosis (Keren *et al.,* 2021).
2. Speed: This method allows for rapid screening and diagnosis, making it suitable for large-scale population screening programs (Riou *et al.,* 2021).
3. Cost-Effective: HPLC is relatively cost-effective compared to more advanced genetic methods, making it accessible in resource-limited settings (Hardison *et al.,* 2020).

Mass Spectrometry (MS)

Advantages:

1. High Resolution and Sensitivity: MS offers high resolution and sensitivity for detecting haemoglobin variants, including those present in low abundance (Higgins, 2020).
2. Detailed Molecular Information: It provides detailed molecular information about haemoglobin structure and composition, aiding in the identification of complex variants (Higgins, 2020).
3. Non-Invasive: MS can be used on dried blood spots, reducing the need for invasive blood draws (Grady *et al.,* 2022).

CRISPR-based detection technology

1. High sensitivity and specificity: CRISPR-Cas9 can detect haemoglobin variants with high accuracy (Chen *et al.,* 2020).
2. Rapid analysis: CRISPR- based detection can be completed in under an hour (Kazazian *et al.,* 2020).

3. Small sample volume: Requires minimal blood sample (Bunn *et al.,* 2020).
4. High-throughput and high accuracy: Can analyse multiple samples in parallel and has high accuracy in detecting haemoglobin variants (Chen *et al.*, 2020).

FUTURE PERSPECTIVES

1. Point-of-care testing: Development of portable, user-friendly devices for rapid detection.
2. Artificial intelligence (AI) integration_: AI-powered analysis for improved accuracy and efficiency.
3. Next-generation sequencing (NGS) advancements: Faster, more affordable NGS for comprehensive genetic analysis.
4. CRISPR-Cas9-based diagnosis: Further development of CRISPR-Cas9 for precise detection and potential treatment.
5. Microfluidic device advancements: Improved microfluidic devices for rapid, low-cost detection.
6. Nanotechnology-based detection: Development of nanoscale devices for ultrasensitive detection.
7. Epigenetic analysis: Investigation of epigenetic factors influencing haemoglobinopathies.

CONCLUSION

Recent advancements in the investigation methods for detecting haemoglobinopathies have significantly enhanced the accuracy, speed, and accessibility of diagnoses. HPLC has emerged as a cornerstone in the detection and quantification of abnormal haemoglobin variants, offering precise and reproducible results. Additionally, capillary electrophoresis (CE) has gained prominence due to its capability to separate a wide range of haemoglobin variants with high resolution, further supported by its automated and rapid analysis features. Molecular techniques, such as PCR and NGS, have revolutionized the genetic analysis of haemoglobinopathies, allowing for the detection of mutations at the DNA level with unprecedented sensitivity and specificity. These advancements collectively contribute to early diagnosis, effective patient management, and the potential for personalized treatment approaches. Despite these technological strides, the integration of these methods in resource-limited settings remains a challenge that necessitates further efforts to ensure global accessibility and equity in healthcare.

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