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SYSTEMATIC REVIEW ARTICLE

The Trajectory of Haptoglobin in Haemolysis, Inflammation and Transfusion Reaction

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Abstract

Background: Haptoglobin is an acute-phase α2-glycoprotein produced in the liver with the major biological function of binding free haemoglobin with very high affinity to prevent the loss of iron following hemolysis. Haptoglobin has an anti-inflammatory property and is raised during inflammation whereas, low level is associated with haemolysis. Transfusion is linked with haemolysis thereby increasing the level of free haemoglobin due to anti-haptoglobin and storage effect. Studies have revealed interplay of haptoglobin in haemolysis, inflammation and transfusion reaction although, the underlying mechanism is not well understood. Besides, its utilization as a diagnostic biomarker and therapeutic advantage have not been well explored hence, this study.

Method: In this review 20 primary studies from various electronic databases such as Google scholar, Semantic scholar and PubMed were obtained on the basis that they were focused on haptoglobin, haptoglobin in haemolysis, inflammation and transfusion. This was made possible by the use of Boolean function.

Results: Haptoglobin, is measured in blood due to its complex formation with hemoglobin, forming a protective non-covalent complex with CD63 as receptor. The finding from this review shows that, haptoglobin plays a crucial role in scavenging surplus hemoglobin, iron and heme in haemolysis with antioxidant function and immunomodulatory effect in transfusion reactions. The concept of the trajectory of haptoglobin explored the multi-dimensional course of this acute phase scavenger protein in the course of clinical conditions of haemolysis, inflammation and transfusion reaction. The review confirmed specific roles of haptoglobin such as physiologic-antioxidant, prognostic, diagnostic biomarker, immunologic and therapeutic. Additionally, an inverse relationship exists between haptoglobin and haemolysis as well as transfusion reaction consequent to hypohaptogloinaemia whereas, direct relationship exists with inflammation resulting to hyperhaptoglobinaemia observed in those clinical conditions respectively. Haptoglobin synthesis is elevated by the liver in response to inflammation,

countering oxidative damage and inflammation by neutralizing free hemoglobin. When there is an immunological mismatch, haemolytic transfusion reactions can occur and transfusion of prolonged stored blood potentiate same effect.

Conclusion: The role of haptoglobin cannot be overemphasized. Based on the widespread roles and clinical relevance of haptoglobin, it is vital that haptoglobin be utilized.

Ket words: Haemolysis, Haptoglobin, Inflammation, Transfusion, Antioxidant

INTRODUCTION

Haptoglobin (Hp) is an acute-phase α_2-glycoprotein produced in the liver with the major biological function of binding free haemoglobin (Hb) with very high affinity to prevent the loss of iron following hemolysis (1,2). Haptoglobin is a multifunctional protein, plays an important role in various biological processes, and is currently considered as a potential biomarker of many diseases, including various forms of malignant neoplasms and has been found in extremely strong non-covalent complex with free Haemoglobin (Hb), which protects tissues from oxidative damage (3). Also, Hp exhibits immunoregulatory properties, participates in the inhibition of nitric oxide, stimulates tissue repair, is involved in angiogenesis, etc. The concentration of Hp in plasma changes with pathology (4).

Haemolysis is a pathological process characterized by the destruction of erythrocytes, leading to the release of cytosolic contents (5). Inflammatory cytokines are produced in the vasculature by hemolysis, and inflammation increases tolerance to free hemoglobin (6). Haemolysis results in a high level of bilirubin, as seen in jaundice (7). Haemolytic anaemia, hyperbilirubinaemia, and infection account for the majority of admissions and readmissions in hospitals. Hp could be used as a guiding indicator to demonstrate the future occurrence of jaundice as well as treat haemolysis (8). Haemolysis can be induced by either intrinsic conditions, in which Red Blood Cell (RBC) presents abnormalities, or by extrinsic circumstances, in which RBC destruction overtakes the bone marrow's capacity for production. Among the intrinsic causes of haemolytic diseases are alterations in haemoglobin (sickle cell disease and thalassemia); metabolic abnormalities (Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency); and RBC membrane instability (hereditary spherocytosis), among others (9). Extrinsic causes of haemolysis, on the other hand, include the development of autoimmune reactions against RBCs e.g. Autoimmune Hemolytic Anaemia (AIHA) and Paroxysmal Nocturnal Haemoglobinuria (PNH)); mismatched transfusion; physical or chemical trauma; infections, such as Plasmodium specie and sepsis (9).

Inflammation involves series of reactions or responses triggered by the presence of an injurious agent like infection, stress, or trauma (10). In response to inflammatory stimuli, such as cytokines (e.g. interleukin-6), the liver increases the production of acute-phase proteins, such as haptoglobin which has anti-inflammatory properties, including the ability to bind and neutralize free hemoglobin released during hemolysis. This helps prevent oxidative damage and inflammation associated with free hemoglobin (11). Haptoglobin, being a plasma glycoprotein and a positive acute-phase reactant (12), is used as an inflammation indicator; this has become important over the last few years. HP could detect inflammation correctly; it is a rapid and sensitive marker of inflammation.

Transfusion is associated with haemolysis thereby increasing the plasma level of free haemoglobin due to the anti-HP. Furthermore long term storage effect explains the haemolytic changes seen in transfusion of stored blood consequent to high level of free haemoglobin. Haptoglobin is linked with anaphalytic transfusion reaction (13).

The trajectory of haptoglobin in Haemolysis, Inflammation, and Transfusion Reactions (HIT) simply explains the course of haptoglobin in physiologic, immunologic conditions which informs its relevant interactions with clinical conditions. The importance of haptoglobin as a good diagnostic biomarker and the characteristic therapeutic effect have not been well known hence, this research. This study delved into the nicety roles of haptoglobin and the trajectory of haptoglobin in Haemolysis, Inflammation, and Transfusion Reactions (HIT), contributing to a deeper understanding of its implications for clinical diagnostics, therapeutics, and transfusion science.

METHODOLOGY

The research is a theoretical paper. In this review, 18 primary studies were included from search results from the following electronic search engines; Google scholar, Semantic scholar and PubMed. This was made possible by the use of Boolean function to narrow the search results to studies relevant to haptoglobin, haemolysis, inflammation and blood transfusion.

Haptoglobin

Synthesis, Structure, Polymorphism and Inheritance of Haptoglobin

Haptoglobin is produced mostly by liver cells (hepatocytes) and other tissues such as kidneys, skin, lungs, and adipose tissue (14). Haptoglobin is synthesized in the liver as an acute phase reactant and by adipocytes, neutrophils and macrophages (15). Production is from a single polypeptide cleaved post-translation into component peptides. The mature protein is formed and then secreted into the plasma. Haptoglobin synthesis by neurons has also been described. Serum Hp has a reference range of 0.3–3 mg/ml, but this varies with phenotype and an individual's level is stable over time under normal circumstances. Increased synthesis occurs as part of the acute phase response, but not in response to low Hp. Variation from individual baseline may indicate haemolysis or inflammation. Synthesis of Hp has been repeatedly shown to be influenced by IL-1, IL-6 and TNF, similar to other acute phase reactants (16).

Haptoglobin (Hp) is made up of four chains: 2 chains (~9kDa each) and 2 chains (~33kDa each). Alpha and beta chains are encoded by a single gene and are synthesized as a single polypeptide chain which is proteolytically cleaved into a short α-chain and a β long chain that is usually connected through a disulfide bond. In addition, an α-β units is linked to another α- β unit also by a disulphide bond [17]. Hp1F, Hp1S, Hp2, controls the formation of six Hp phenotypes: 1F-1F, 1S-1S, 1F-1S, 2-1F, 2-1S, 2-2. Due to the lack of a functional difference between Hp1F and Hp1S, which differ only in point mutations, only two alleles, Hp1 and Hp2, are often considered, which manifest themselves as three phenotypes: homozygous Hp1-1 and Hp2-2, and heterozygous Hp2-1 depending on the combination of inherited allelic variants. It is assumed that the Hp2 allele was created as a result of intragenic

duplication of a 1700 bp DNA fragment of gene Hp1 after human divergence in the late evolution of primates (18).

In humans, Hp is characterized by a genetic polymorphism which arises from differences in α-chains and the β-chains are often identical in all Hp types. The Hp locus is located on chromosome 16 (16q22.1). Hp is made up of two alleles, Hp1 and Hp2 that give rise to three major phenotypes. Individuals that are homozygous for allele Hp1 express the phenotype 1-1, those homozygous for allele Hp2, express phenotype Hp2-2, and heterozygous individuals express phenotype Hp1-2. Hp1 allele is often organized in 5 exons, the first 4 exons encode for α subunit while exon 5 encodes for β subunit. Hp2 allele is made up of 7 exons, the first 6 exons encode usually for larger form of α-subunit and exon 7 encodes for β-subunit. The larger form of the Hp α-subunit seems to originate from an intragenic duplication of exons 3 and 4. As a consequence, Hp1-1 phenotype is made up of homodimers of two α-β units, but Hp1-2 and Hp2-2 consist of polymers, as the cysteine that forms the disulfide bond between α-subunits is duplicated in Hp2. The resultant stoichiometry is for Hp1-1 homodimers of (α1-β)2; for Hp2-1 linear polymers of(α1-β)2 + (α2+ β)n (n=0,1,2, etc); and for Hp2-2 cyclic polymers (α2+ β)n (n=3,4 etc) (Van *et al*., 2004).

Binding of Haptoglobin to Haemoglobin

The binding of Hb to Hp1-1 leads to the formation of an approximate 160 kDa complex. Much larger complexes are formed, when Hb binds to the Hp2-1 and Hp2-2 forms. Whatever kind of Hb-Hp complex is formed, the complex formation effectively reduces renal filtration of Hb. In addition, it elicits a high affinity site for CD163 recognition leading to clearance of Hp and Hb (26). As a consequence, hemolysis leads to consumption of Hp that can be virtually absent, if the release of Hb into plasma overrides the production of the Hp. A low Hp level in plasma is therefore a strong and well-known biomarker for accelerated intravascular hemolysis. Despite circulating Hp in its free none-Hb-bound form does not bind to CD163, the Hb-bound Hp is directly involved in the binding to CD163 (20).

Role of Haptoglobin

Physiologic Role

Haptoglobin has physiological roles other than the metabolism of hemoglobin. Locally synthesized haptoglobin may provide antioxidant and antimicrobial effects. Anti-oxidative, bind, scavenge, or neutralize free Hb, and prevents hemoglobin-mediated renal injury and iron loss following hemolysis (21). These multiple functions of haptoglobin stated above including, CD163 adaptor functions, detoxifying haptoglobin, and prevention of haem release from ferric (Fe^{3+}) haemoglobin. Due to its unique anatomic location, the vascular wall appears to be the principal target of free haemoglobin exposure during haemolysis. Nitric oxide consumption by free haemoglobin triggers endothelial inflammatory activation, which is the principal pathophysiologic component that stimulates the disease process as seen in cardiovascular disease (10). The major-characterized function of Hp is intravascular sequestration of extracellular or free haemoglobin following the formation of large Hb-Hp protein complexes, a process that prevents extravasation of free haemoglobin into tissues. This effect is particularly evident in the kidneys, where oxidative reactions at the haeme moiety of Hb lead to globin deposition (hyaline casts), iron overload, lipid peroxidation and renal tubular injury (21). Haemolytic stress causes renal injury due to oxidative damage. Haptoglobin is protective by irreversibly binding free hemoglobin. Haemoglobin-haptoglobin complexes are rapidly cleared from circulation by monocytes and tissue macrophages via CD163 receptors (21).

Haptoglobin as a Biomarker

Haptoglobin serves as diagnostic biomarker for assessment of some clinical conditions, including haemolysis, inflammation, and transfusion reactions; (22) also in oxidative stress, anaemia and others. Haptoglobin is low in patients with increased haemolysis, irrespective of whether haemolysis occurred intravascularly or extravascularly. Several studies have shown reduced HP in haemolytic states, and the determination of haptoglobins in serum is thus of potential value in the detection of haemolysis. Notably, haemolysis has a substantial contribution in hyperbilirubinaemia. An inverse relationship exists between haptoglobin and jaundice, haptoglobin is an indicator in the early detection of jaundice (21, 23-25). Also, haptoglobin is recognized as an independent prognostic marker of ovarian and breast cancer, there is a significant increase in the level of Hp in plasma compared with individual not having ovarian cancer (26-27).

Immunologic Role

Suppressed lymphocyte proliferation, including B-cell mitogenesis; alters the TH-cell distribution and modulate immune system have been reported (22). Haptoglobin can suppress proliferation of lymphocytes and B-cell mitogenesis, as well as modulating macrophage function by inhibiting viral hemagglutination and prostaglandin H synthase. By altering the distribution of helper T-cells, haptoglobin can function as an immune system modulator and may be partially responsible for certain infections, allergies, and autoimmune disorders. Haptoglobin has also been found to induce angiogenesis, most notably in the Hp2-2 subtype (21, 23-25).

Therapeutic Role

Hp is useful in the treatment of shock, haemolysis, hypotension, and prevents kidney injury (25). Haptoglobin has been proven to bind extracellular (free) haemoglobin, which is a toxic product of haemolysis. Component transfusion such as red cell concentrate or stored whole blood, is associated with haemolysis on storage (prolonged), thereby increasing the plasma level of cell-free haemoglobin. Study confirmed that treatment with exogenous human haptoglobin possess an ameliorating potential for resuscitation with stored red blood cells (SRBCs) after 2 hours of haemorrhagic shock in mice by improving the survival rate and attenuated SRBC-induced inflammation. Treatment with haptoglobin retained free haemoglobin in the plasma and prevented SRBC-induced haemoglobinuria and kidney injury (23).

Haptoglobin and Haemolysis

Hemolysis is a pathological process characterized by the premature loss of red blood cell membrane integrity leading to the release of the cytosolic content, mainly comprised of haemoglobin, in the extracellular space. It can be triggered by various pathological factors, including genetic abnormalities of haemoglobin (sickle cell disease and β-thalassemia), complement regulators (Paroxysmal Nocturnal Haemoglobinuria and atypical hemolytic uremic syndrome), pathogens (malaria, sepsis, and typical hemolytic uremic syndrome), auto- or alloantibodies, oxidative stress, toxins, trauma, or blood transfusion (27). Based on the capability of the bone marrow to put up a compensatory mechanism or not, haemolytic anaemia is either classified as compensated or uncompensated hemolytic anaemia. Haemolysis may be acute, chronic, or episodic (27). Also, haemolysis can be classified according to whether the haemolysis is outside (extrinsic) or within the red blood cell (intrinsic) (28).

A link exists between haptoglobin and haemolysis. Haptoglobin decreases with increased haemolysis, irrespective of whether haemolysis occurred intravascularly or extravascularly although some have argued this stating that it is mainly intravascular

haemolysis (29). Also several studies have shown reduced HP in haemolytic states and haemolysis associated conditions such as jaundice (30-32). There are clinical effects associated with haemolysis. High mortality especially in SCD, infection including toxic shock. Furthermore, hemolysis-associated renal failure in this patient cohort was also inversely associated with Hp plasma concentrations (25). Jaundice is an adverse effect of haemolysis and haptoglobin is linked with jaundice. An indirect correlation exists between HP and jaundice, HP is an indicator in the early detection of jaundice In jaundice, the basic problem is the imbalance between the rate of production due to haemolysis and the elimination of bilirubin in less developed or inactive liver (33). Haemolysis has a significant role in bilirubin increase (34).

The determination of haptoglobins in serum is thus, of potential value in the detection of haemolysis and the advent of the application of haptoglobin in case management of haemolysis makes Hp a useful therapeutic tool in modern medicine.

Furthermore, the clinical implications of haemolysis cannot be overemphasized and there are other clinical conditions associated with haemolysis including inflammation and other immunomodulatory induced conditions.

Haemolysis and inflammation

Haemolysis shares an association with inflammation. Haemolysis induces production of inflammatory cytokines by neutrophils and monocytes in the vascular microenvironment. Haemolytic conditions like Sickle Cell Disease (SCD), is one of such. The inflammatory cytokine and chemokine landscape of SCD patients in different statuses confirms that SCD is associated with proinflammatory profile (39).

Implications for patient of the concept of haemolysis in inflammation remains key as the understanding will manage patients and provide an improved treatment outcome. Remarkably, haemolytic diseases are associated with thrombosis, inflammation and immune dysregulation, all together contributing to organ damage and poor outcomes. Furthermore, haemolysis results to anaemia, loss of the anti-inflammatory functions of red blood cells, release of damage-associated molecular patterns including ADP, haemoglobin, and haeme which act through multiple receptors and signalling pathways, fostering a hyperinflammatory and hypercoagulable state (40).

Clinical Implications of the Trajectory of Haptoglobin in Haemolysis

Haemolytic stress causes renal injury due to oxidative damage, Hp serves as remedy through its anti-oxidant effect. Also, Haemolytic stress causes inflammation of liver, cirrhosis, and splenomegaly due to RBC accumulation and adhesion, this can be averted by Hp. Remarkably, haptoglobin has a detoxifying ability that ameliorate adverse effect of haemolytic conditions in patients. Besides, haptoglobin is protective by irreversibly binding free haemoglobin, which retains the iron that is needed by the body (35-38). Furthermore, high mortality especially in SCD, infection including toxic shock. Furthermore, hemolysis-associated renal failure in patients is also inversely associated with haptoglobin plasma concentrations as revealed in a study (36).

Haptoglobin and Inflammation

Inflammation is the natural response of the body to perceived harm. It is also one of the most potent healing mechanisms to manage various infections, injuries, and stress. Inflammation can be categorized as either acute or chronic (41). Acute inflammation is a five-stage process, namely: heat or burn, pain, swelling, redness, and loss of function.

A study shows haptoglobin activity is upregulated by 2–10 times during acute-phase response processes such inflammation and tissue damage (42). The same is true in malignancies, atherosclerosis, type I diabetes, inflammatory bowel diseases and several autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis (43). Secretion of Hp could be strongly induced by pro-inflammatory cytokines such as interleukin (IL)-6, IL-1 and tumor necrosis factor (TNF)-α. in this regard, it seems feasible that high HP levels may be a consequence of activation of multiple inflammatory pathways in autoimmune diseases like rheumatoid arthritis (31, 44).

Additional, Inflammation describes the processes involved in the disturbance of tissue homeostasis as a result of acute or chronic stimuli from an infection, stress, autoimmune raction or mechanical injury (44). The homeostatic immune surveillance is largely mediated by polymorphonuclear leucocytes (PMN), with the disturbance eliciting PMN migration through the TH1/TH2 cytokine profile (45). Hp actively participates in all the processes from PMN recruitment and free radical quenching, to tissue repair and regeneration. The reduction or absence of Hp protein, as seen in hypohaptoglobinemia or ahaptoglobinemia, is associated with allergic (skin and lungs) and anaphylactic transfusion reactions, respectively (45-47).

Haptoglobin and Transfusion Reactions

Transfusion reaction is any adverse reaction associated with transfusion of blood or blood products. Transfusion reaction have different classifications based on immunologic process and onset (45). See figure for details.

In transfusion medicine, the most imperative issue about haptoglobin is perhaps anaphylactic transfusion reactions. Individuals who are genetically deficient in haptoglobin and who carry the anti-hp antibody may experience adverse transfusion reactions against the Hp protein in blood components and also, transfusion of stored red cells especially prolonged storage (10). Transfusion reaction can be managed by: discontinuing/ stopping the transfusion, administration of corticosteroids, intravenous immunoglobin, and rituximab (for alloimmunization), and others. Notably, haptoglobin has been shown to have therapeutic effect in managing transfusion associated haemolysis (47-50).

The Trajectory of Haptoglobin in Haemolysis Inflammation and Transfusion Reaction

The concept of the trajectory of haptoglobin in haemolysis, inflammation and transfusion reaction demonstrates the interplay of the multi-purpose scavenger acute phase protein (haptoglobin) in the course of these clinical conditions (haemolysis, inflammation and transfusion reaction).

Haptoglobin is a sensitive marker for hemolytic conditions like anaemia, and haemolysis it is reduced, demonstrating an inverse relationship. On the other hand, haptoglobin is an acute-phase reactant, elevated in infection, inflammatory disease, or other reactive states (21). The intracellular contents of the red cells are liberated into the vascular system, leading to various adverse reactions such as oxidation-oxidative stress, inflammation and platelet aggregation (24). Due to its unique anatomic location, the vascular wall appears to be the principal target of free Hb exposure during hemolysis. Haemolysis is associated with the release of free haemoglobin and these free haemoglobin in-turn consume nitric oxide.

Nitric oxide consumption by free Hb triggers endothelial inflammatory activation and this is the principal pathophysiologic component that stimulates the disease process seen in CVD. The mechanism of platelet aggregation is based on the fact that the free haemoglobin released to the vasculature scavenges nitric oxide (NO) in the endothelium, thereby causing vasoconstriction and reduced blood flow. Besides, free haemoglobin can cause

direct cytotoxic injury to cell membranes, plasma proteins, and lipids (24). Furthermore, high concentrations of free haemoglobin in the plasma have been observed to be linked with direct organ injuries, including renal failure, intestinal mucosal damage, or lung injury (24-25, 36). Haptoglobin level was completely protective against haemoglobinuria and hypertension during an 8-h infusion of free haemoglobin. Inflammation appears to enhance tolerance against free Hb (21).

The Hb-Hp scavenger pathway's activity is influenced by inflammatory and anti-inflammatory processes on several levels (38). Systemic inflammation, particularly if it involves the interleukin (IL)-6 effector pathway, increases expression of Hp in the liver and many parenchymal and non-parenchymal cells. IL-6 has also been reported to enhance expression of CD163 on macrophages, suggesting that enhanced Hb sequestration and clearance capacity are general adaptive responses to infection and tissue injury (21). Intriguingly, however, some inflammatory mediators, such as endotoxin and other Toll-like receptor (TLR) agonists or tumor necrosis factor-α (TNF-α) trigger protease-mediated shedding of CD163 from the cell surface of monocytes and macrophages. This shedding acutely blocks clearance of Hb-Hp complexes by monocytes (38). High levels of soluble CD163 are consequently found in patients with sepsis or more specific macrophage activation syndromes. Regulation of the Hb clearance system by anti-inflammatory glucocorticoids is evident. In an experiment model, high Hp level was completely protective against haemoglobinuria and hypertension during an 8-h infusion of free Hb. Inflammation appears to enhance tolerance against free Hb (28).

Basically, haemolysis is one of the adverse effects of transfusion reaction and inflammation have been linked with haemolysis. Also, haptoglobin is connected with inflammation possessing anti-inflammatory function. These interwoven conceptions necessitate the significance and clinical implications of haptoglobin in HIT. Remarkably, the concept of the trajectory of haptoglobin explores the multi-dimensional course of this acute phase scavenger protein in the course of clinical conditions of haemolysis, inflammation and transfusion reaction. An inverse relationship exists between haptoglobin and haemolysis as well as transfusion reaction consequent to hypohaptogloinaemia whereas, direct relationship exists with inflammation resulting to hyperhaptoglobinaemia observed in those clinical conditions respectively. Haptoglobin synthesis is elevated by the liver in response to inflammation, countering oxidative damage and inflammation by neutralizing free hemoglobin. When there is an immunological mismatch, haemolytic transfusion reactions can occur and transfusion of prolonged stored blood can potentiate same effect.

Diagnosis of Haptoglobin

Haptoglobin have proven to be a good biomarker used for assessments in clinical conditions. Haptoglobin investigations may be ordered based on symptoms like fatigue, pale skin, fainting, shortness of breath, rapid heart rate, jaundice, and unusual urine color. Testing is also conducted when laboratory results suggest hemolytic anemia, transfusion reaction, or inflammation, verifying these conditions through haptoglobin levels (46-48).

As a biomarker for haemolysis, jaundice, anaphylytic transfusion reaction and inflammation can be performed using different methods)46-48].

Haptoglobin phenotyping

As earlier mentioned, haptoglobin types influence the chemical structure of the products of the gene. Individuals homozygous for the Hp1 allele (Hp 1-1 phenotype) have only Hp 1 dimers in their serum, and individuals harboring 2 Hp2 alleles (Hp 2-2 phenotype) bear Hp 2 polymers with various sizes. Heterozygotes with both Hp1 and Hp2 alleles have Hp 1 dimers and Hp 2-1 polymers as well. These proteins can be separated by gel electrophoresis, isoelectric focusing, chromatography, or ELISA. A typical diagram of electrophoresis results is shown in the figure below. Though these phenotyping methods have been used for a relatively long time and many studies have been conducted based on these methods, they require special equipment and experienced personnel to interpret the results. Additionally, these techniques are not designed to detect patients harboring the Hp^{del} allele that is, they cannot differentiate true anhaptoglobinemia from conditions of acquired undetectable haptoglobin levels (48).

Each patient serum is run in two adjacent lanes. The first lane detects presence of free hemoglobin, hemopexin-hemoglobin complexes, and methemalbumin. The presence of haptoglobin-hemoglobin complexes is ignored in this lane as any degree of non-pathological hemolysis will result in presence of a protein band. The second lane is one volume of patient plasma mixed with one volume of known free Hb concentration (60 mg/dl) incubated together at room temperature for 30 min. If there is no presence of the free hemoglobin band, the patient serum haptoglobin concentration must be at least 30 mg/dl or higher. If haptoglobin is reduced, free hemoglobin and haptoglobin-hemoglobin will be present on the 1:1 mix lane. If it is absent, only the free hemoglobin will be present (50-51). Also, in the figure above B represent an example gel. Controls for components of the hemolytic screen are run to the far right of the gel.

Haptoglobin Genotyping
Southern Blotting

As the genetic structures of Hp1 and Hp2 alleles were revealed, many researchers have tried to determine haptoglobin genotypes using molecular genetic techniques. Using various restriction enzymes and probes, Southern blotting has been effectively used to determine haptoglobin genotypes. However, this approach is not free from the limitations inherent to the method itself—requirement of a large amount of genomic DNA labor and time consumption and risk of radiation hazards. As more convenient and safe genotyping methods are being developed, the utility of Southern blotting has been steadily decreasing (52).

Conventional Polymerase Chain Reaction (PCR)

Koda et al. used conventional PCR for detecting Hp^{del} allele. They targeted the junction region of Hp^{del} allele to produce an amplicon of 315 bp. Exon 1 of the Hp gene was also amplified as a control (476 bp). The combination of these 2 products can identify individuals homozygous for Hp^{del} (315 bp only), heterozygous for Hp^{del} (315 and 476 bp), and without Hp^{del} (476 bp only). However, this strategy cannot distinguish between Hp1 and Hp2 alleles (10).

Genotyping methods using conventional strategies for determining the Hp1 and Hp2 alleles can be achieved by using 4 primers simultaneously to distinguish Hp1 from Hp2 alleles this was suggested by Olatunya *et al*. (53). Appropriate combinations of various conventional PCR methods can successfully detect the various combinations of the Hp alleles. Nevertheless, genotyping strategies using conventional PCR require keeping multiple sets of primers and performing tedious post-amplification processes, such as electrophoresis. In addition, it is difficult to detect relatively large products over 3 kb, especially in poor amplification conditions. Typical patterns of a conventional PCR corresponding to specific haptoglobin genotypes are shown in Figure below (53).

Real-Time Polymerase Chain Reaction (PCR)

To overcome the drawbacks of conventional PCR, Olatunya et al. (53) developed a haptoglobin genotyping strategy using real-time PCR. According to the typing purpose, two types of detection techniques were used.

Loop-Mediated Isothermal Amplification (LAMP)

This method is one of the most recently developed and applied in many fields. This method can amplify nucleic acids with high degree of sensitivity and specificity in isothermal condition, requiring only a simple heating block or water bath. And a positive reaction can be detected by a simple visual inspection of turbidity. Michiyuki et al. (48) have developed LAMP method for detection of Hp^{del} allele. This method can efficiently analyze few samples without a sophisticated thermal cycler and detection apparatus. But two reaction tubes are required, and it cannot differentiate Hp1 and Hp2 alleles.

Advantages and Disadvantages of Diagnostic Techniques

Spectrophotometric methods, measuring light absorption at various wavelengths in serum with a reducing agent, determine haptoglobin-hemoglobin concentrations. However, interference from substances like bilirubin and chylomicrons affects accuracy. Note that distinguishing hemoglobin-haptoglobin complexes from free haptoglobin varies between spectrophotometric and immunological methods (21). See table 1 for details.

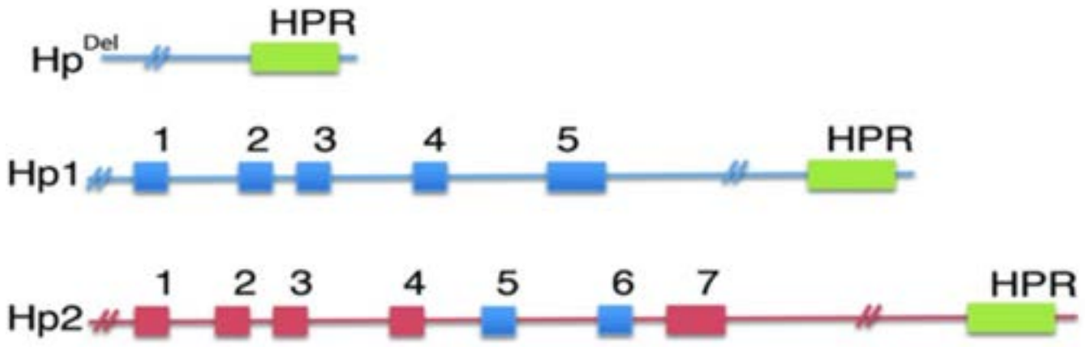

Figure 1: Structural representation of the Hp alleles Del, 1, and 2 [18]. Hp exonic sequences are denoted by numbered and shaded boxes. Intronic sequences are denoted by a solid line. Exons 3 and 4 of the Hp1 allele have been duplicated in the Hp2 allele, giving rise to exons 3–6 [19]

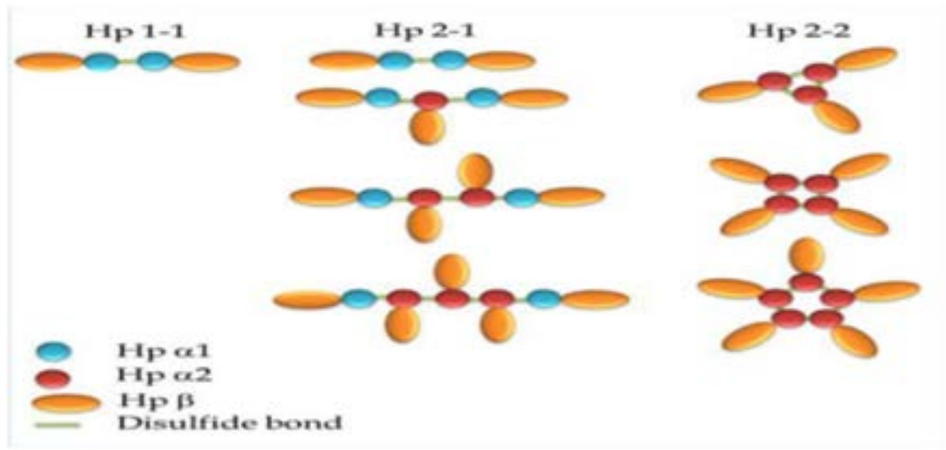

Figure 2 Schematic representation of the structure of the different haptoglobin polymers determined by phenotype (19).

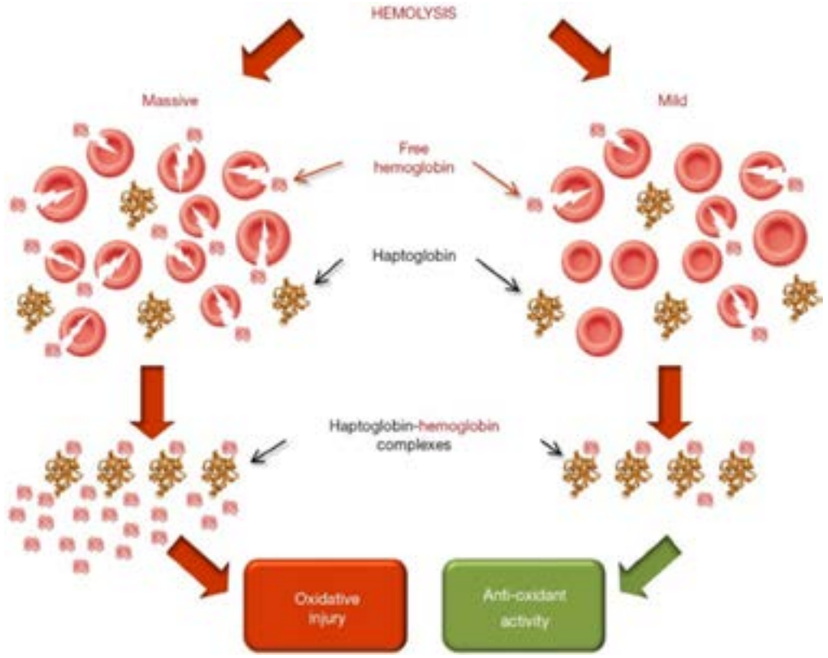

Figure 3: Formation of Haptoglobin-Haemoglobin Complex (35-38).

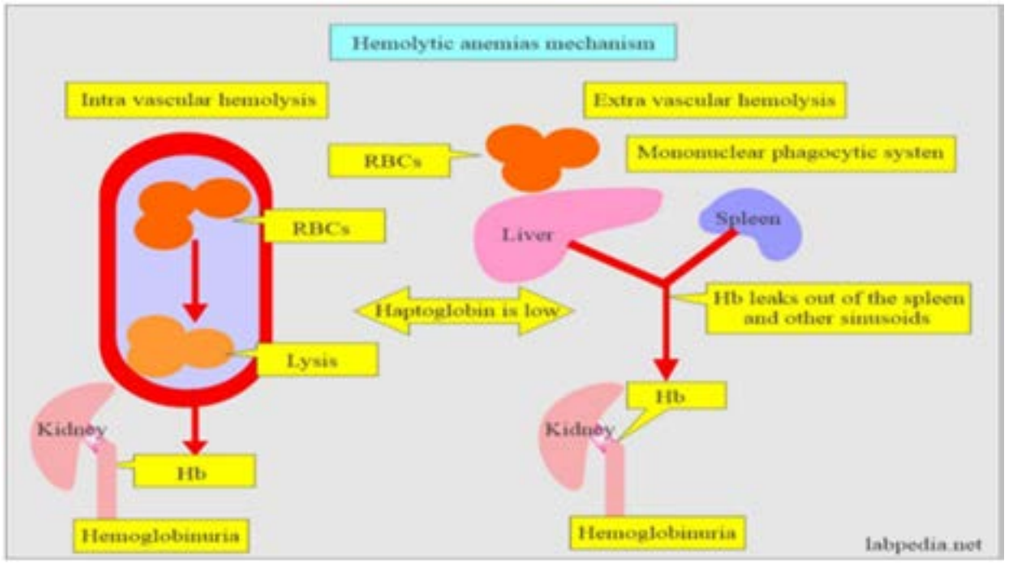

Figure 4: Haptoglobin (HP) and Hemoglobin (Hb) complex role in inflammation and hemolysis (22).

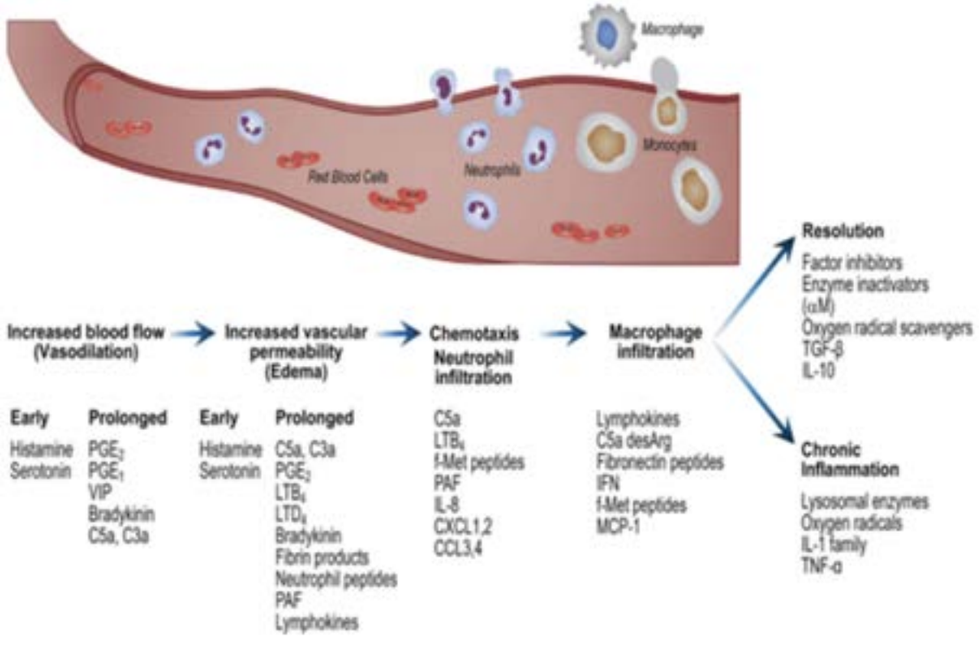

Figure 5: Representation of Inflammation (35, 36).

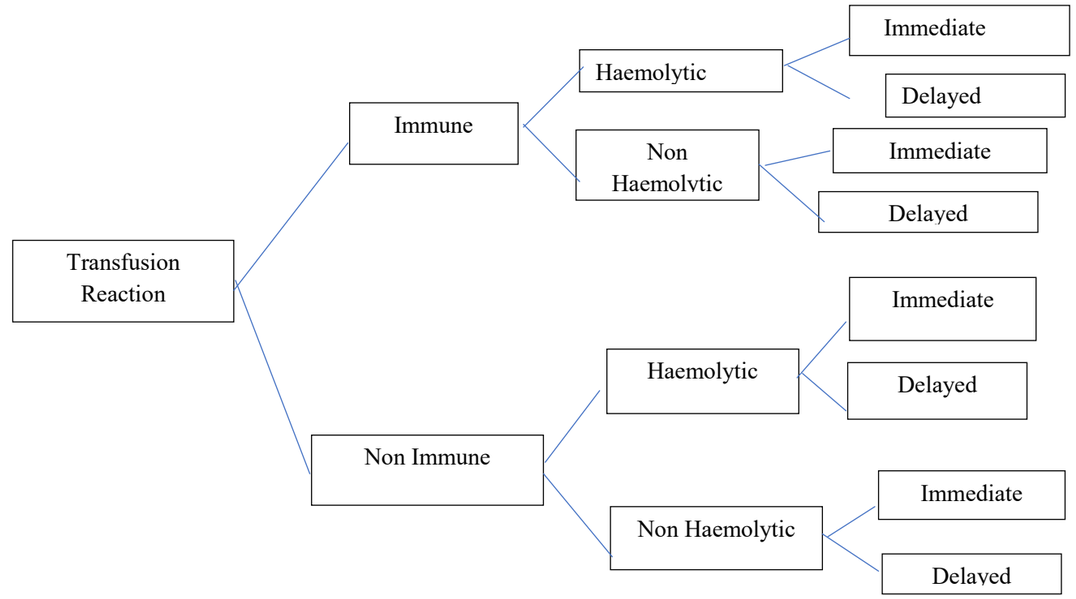

Figure 6: Classification of Blood Transfusion Reaction. (46)

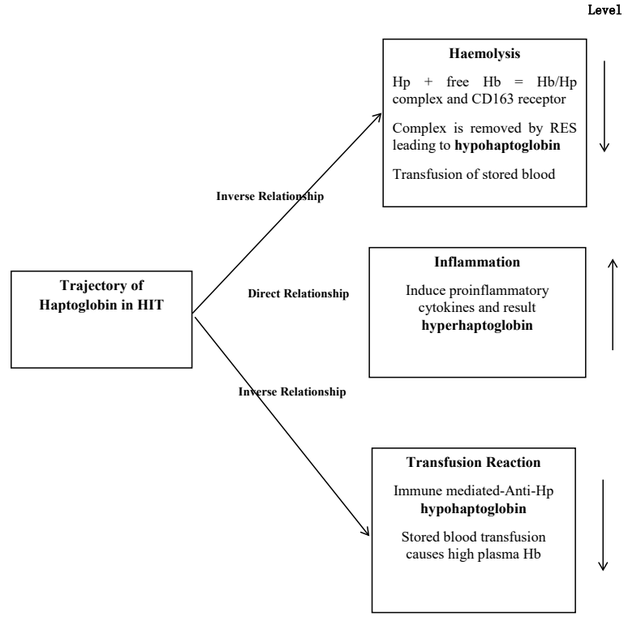

Figure 7: Research Framework of the Concept of the Trajectory of Haptoglobin in Haemolysis, Inflammation and Transfusion Reaction (50). Note: HIT means Haemolysis, Inflammation and Transfusion Reaction, RES means Reticulondothelial System

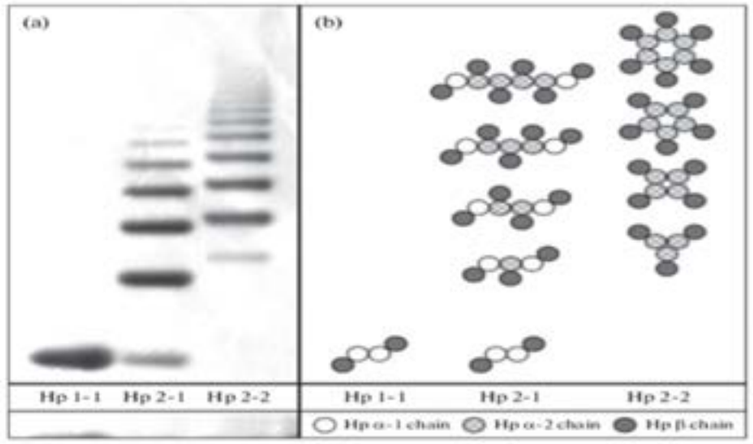

Figure 8: Haptoglobin phenotype assessment by native PAGE. (a) Particular profiles produced by gradient (3–8%) native PAGE electrophoresis of haptoglobin preparations with different morphologies. (b) The composition of the three haptoglobin phenotypic polymers: Hp1-1 homodimers, Hp2-1 linear heterodimers, and Hp2-2 cyclic heterodimers (3).

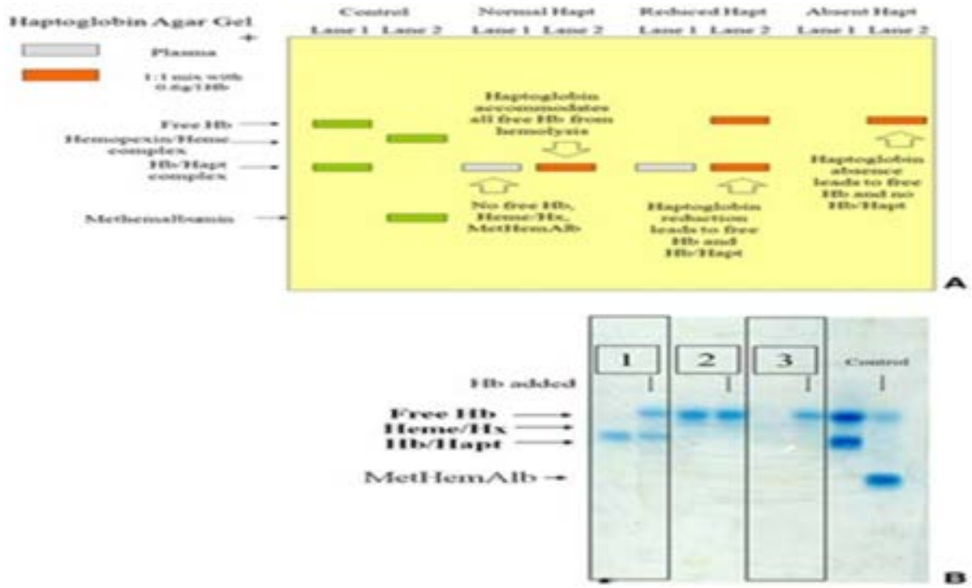

Figure 9: A. A. cartoon illustration of an agar gel containing haptoglobin. Serum from each patient is run in two parallel channels. Hemopexin-hemoglobin complexes, methemalbumin, and free hemoglobin are all detected in the first lane. B. A sample gel. The hemolytic screen's controls are located much to the right of the gel. (50).

Enyzme Linked Immunosorbient Assay (ELISA) Technique

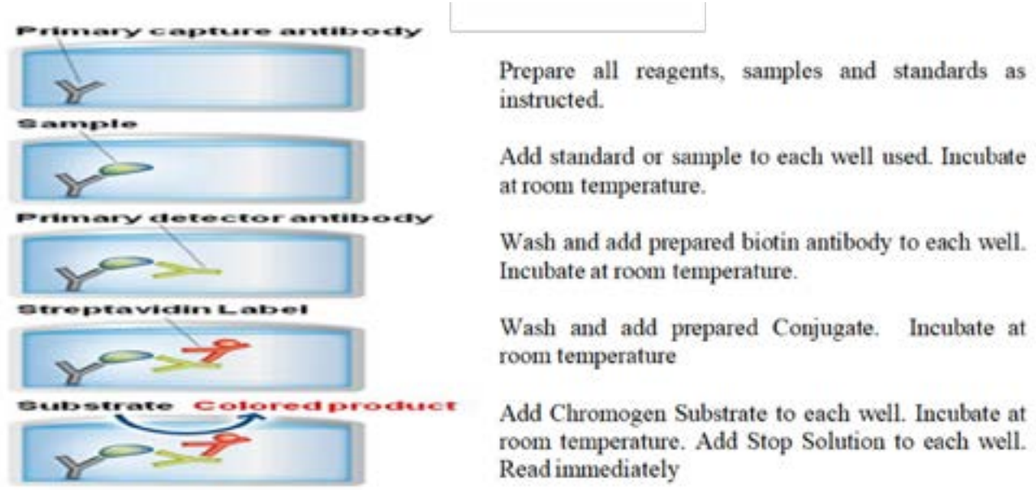

Figure 10 : Schematic Steps of laboratory procedure of testing haptoglobin using ELISA method

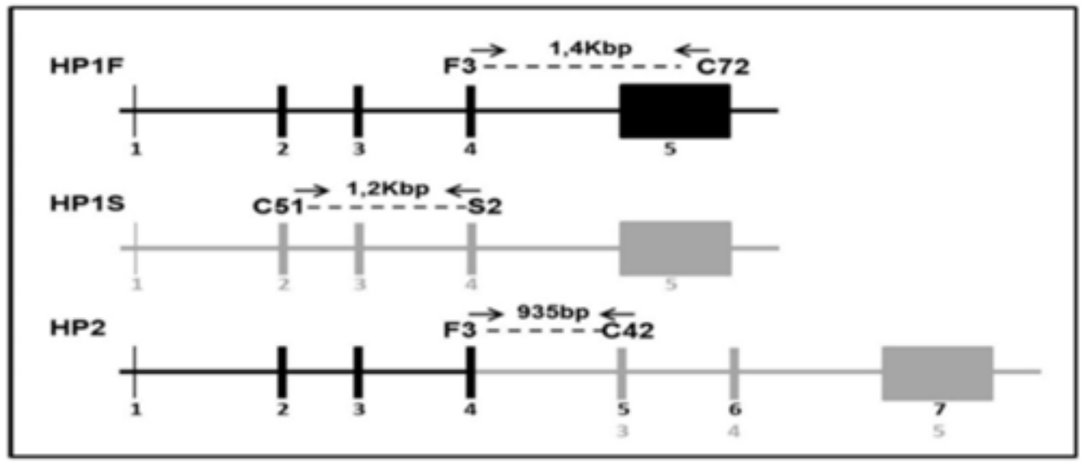

Figure 11: Allele-specific PCR is a method of selectively amplifying the many HP alleles [53].

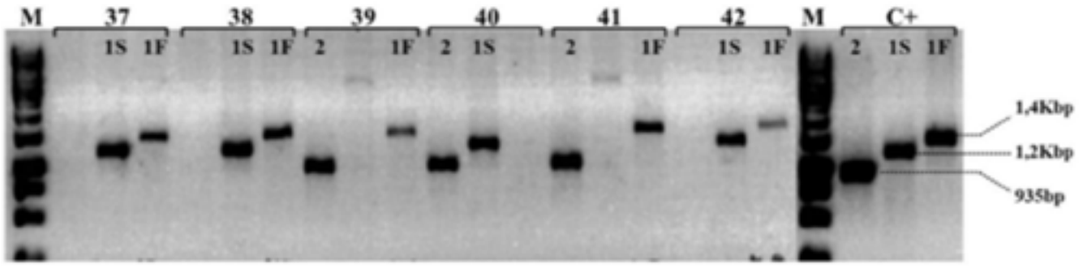

Figure 12: Genotyping of hemoglobin using allele-specific PCR. (53).

Table 1: Summary of the Principles and Characteristics of Various Assays for Haptoglobin

Method	Typing principle	Advantages	Disadvantages
Phenotyping [42, 47]	Structure and size variations in proteins	Used for a long time Large amount of data accumulated Detects rare and/or new variants	Cannot detect genotype Requires special equipment and trained Personnel
Southern blotting [45]	Restriction size variation	Detects Hp^{del} allele May recognize new alleles	Labor and time consuming Large amount of DNA Risk of radiation hazard
Conventional PCR (Olatunya et al., 2020; Gulhar et al., 2023).	Size variation of amplified products	Differential. Distinguishes between different alleles under appropriate combinations	Need to keep multiple primer sets Tedious post amplification process Difficult to amplify and detect large-sized products
Real-time PCR using TaqMan probe [46]	Signals from probes reacting to amplified regions and their ratios	Discriminates between different alleles in a single reaction	Cannot detect rare variants Multiple sets of primers and probes Reaction failure in a large-scale study.
Real-time PCR using SYBR Green I [46].	Melting curve analysis	Detect Hp^{del} allele effectively	Cannot distinguish between and Reaction failure in a large-scale study.
Loop-mediated isothermal amplification [41].	Turbidity measurement	Detect Hp^{del} allele effectively No need for a thermal cycler	Multiple sets of primers and 2 reaction tubes needed Not thoroughly evaluated Cannot differentiate

Conclusion and Recommendation

Clinically, haptoglobin assessment is crucial for diagnosing and treating conditions like anemia, oxidative stress, and other illnesses. It also modulates the immune system and proves beneficial in managing hemolysis and preventing adverse effects of transfusion-related complications. Clinicians and medical practice globally should consider utilization of this multifunctional tools and harness its benefits.

Haptoglobin is an essential biomarker, signaling hemolysis and jaundice with decreased levels due to free hemoglobin. Increased haptoglobin indicates infection or inflammation, and it may contribute to anaphylactic transfusion reactions. Understanding and correctly interpreting haptoglobin test results is vital for clinicians and laboratory scientists.

Limitation

This paper is a theoretic paper and also limited by number of articles reviewed hence, further empirical and more in-depth reviews are recommended.

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Conflict of Interest

None

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