ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

GENETIC ASSOCIATION AND POLYMORPHISMS OF HAPTOGLOBIN AND ABO BLOOD GROUPING IN SOME LEPROSY PATIENTS IN ETHIOPIA

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Genetics)

By

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Addis Ababa, Ethiopia

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Abbreviations

AFB	Acid fast bacilli
AHRI	Armauer Hansen Research Institute
ALERT	All African Leprosy Tuberculosis Rehabilitation Research Training Center
BB	Borderline
BL	Borderline Lepromatous
BT	Borderline Tuberculoid
CVD	Cardio-vascular Disease
EL	Erythro-lysis
ELISA	Enzyme Linked Immune Sorbent Assay
ENL	Erythema Nodosum Leprosum
GWAS	Genome Wide Association Study
Hb	Hemoglobin
Hp	Haptoglobin
Hpr	Haptoglobin related protein
IFN	Interferon
IL	Interleukin
KL	Karyo-lysis
LL	Lepromatous Leprosy
MB	Multi-bacillary

NK	Natural Killer cells
PB	Pauci-bacillary
PCR	Polymerase Chain Reaction
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
SNP	Single Nucleotide Polymorphism
TNF	Tumor Necrosis Factor
TT	Tuberculoid
WHO	World Health Organization

Abstract

Haptoglobin, which is encoded by the Hp gene located on chromosome 16q22, is hemoglobin-binding protein. It has antioxidant and immune-modulatory properties which are crucial for innate immune response. The common Hp gene polymorphism consists of two structurally different alleles (Hp1 and Hp2) and in some rare case its deletion (Hp0). There are clear functional differences between the genotypes which include differences in modulation of oxidant stress, recycling of hem-iron, and immune functions. These functional differences associate with the risk of several infectious and non-infectious diseases. This study aimed to assess the Hp genotype frequency and ABO blood group distribution pattern among leprosy patients from ALERT Referral Hospital in Ethiopia. In the study about 2-3mL venous blood samples were collected from 109 leprosy patients and 108 healthy controls. ABO blood groups were determined by slide technique using commercially available anti-A and anti-B monoclonal antibodies. DNA was extracted by simple salt out method from Buffy coat and Hp genotypes were determined by PCR reaction. The plasma haptoglobin levels were determined by ELISA technique. Data were analyzed using SPSS version 21 and STATA version 12.1. P-values less than 0.05 were considered as statistically significant. This study indicated that the distribution of ABO blood types in leprosy patients was 29.4%, 22.9%, 4.59% and 43.1% for blood type A, B, AB and O, respectively. This distribution was not significantly differ from the healthy controls (p=0.638). However, there was significant association between ABO blood group and mid-borderline leprosy (p =0.016). The mean of plasma haptoglobin concentration in leprosy patients was 1.14 ± 0.62mg/ml with median of 1.02mg/l and this was not significantly different from healthy controls (P = 0.138). However, the concentration was increasing along leprosy spectrum with non-significant difference. The genotypic frequencies of Hp1-1 (6.35%), Hp1-2 (76.19%), and Hp2-2 (17.46%) in leprosy patients were significantly different from healthy controls [Hp1-1 (4.76%), Hp1-2 (92.06%), and Hp2-2 (3.18%)] (P = 0.019). The Hp2 allele was more frequent in leprosy patients, but this difference was not significant.

Key words: *Concentration, Genotype, Haptoglobin, Leprosy, Phenotype*

Declaration

I, the undersigned, declare that this thesis is my original work. It has not been submitted for any another degree or qualification in Addis Ababa University or any other university or other institute. All sources of materials used for the thesis have been acknowledged.

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Signature: ----------

Date of submission: *June, 2014*

Place: *Addis Ababa, Ethiopia*

1. Introduction

Haptoglobin (Hp) is an acute phase hemoglobin-binding plasma glycoprotein (Sadrzadeh and Bozorgmehr, 2004) which scavenges hemoglobin released into the circulation by hemolysis or normal red blood cell (RBC) turnover. It is composed of two α chain and two β chain proteins connected by disulfide bridges. The α-chains of the Hp2 protein are composed of 142 amino acids residue whereas the two α chains of Hp1 are made up of 83 amino acids residue.

The haptoglobin protein is encoded by a gene located on chromosome 16q22 consisting of two common co-dominant alleles [Hp1 and Hp2] which code for different α-chains but identical β-chains (Maeda and Smithies, 1986). The Hp2 allele, with its intragenic duplication, codes for an α-chain that is almost twice as long as those coded by the HP1 alleles (Maeda and Smithies, 1986). Although Hp gene principally expressed in liver, it is also expressed in other tissues such as lung, skin, spleen, kidney, and adipose tissue (Sadrzadeh and Bozorgmehr, 2004).

Besides its hemoglobin scavenging role, Haptoglobin has many biological functions. In particular, it is needed as an immune system regulatory compartment development (Huntoon et al., 2008; Quaye, 2008) which mainly involve in immune response in leprosy. Foremost, helper T-cell type 1 (Th1) and helper T-cell type 2 (Th2) are the two groups of T lymphocytes that regulate cellular and humoral responses (Sadrzadeh and Bozorgmehr, 2004). These two polar immune responses are the major immunological response for leprosy disease (Walker and Lockwood, 2006) and they are balanced by haptoglobin (Huntoon et al., 2008; Quaye, 2008). Furthermore, haptoglobin also involves in oxidative stress prevention, anti-oxidant function, and anti-inflammation functions.

Several studies associated haptoglobin gene polymorphism with the prevalence of different types of diseases such as infectious diseases, cardiovascular diseases, and hematologic disorders (Sadrzadeh and Bozorgmehr, 2004). Infectious diseases associated to haptoglobin include AIDS_ Hp2-2, TB_ Hp2-2, hepatitis_ Hp1-1, and malaria_ Hp2-2 phenotype is protective against severe *Plasmodium falciparium* (Quaye, 2008). Studies on haptoglobin

have shown the susceptibility or resistance of individuals to many non-infectious diseases based on the haptoglobin phenotype. Some of these include; myocardial infarction_ Hp 2-2 phenotype, diabetes_ Hp 1-1 phenotype, hematologic disorders_ Hp1-1, and hypertension_ Hp1-1 (Miranda-Vilela et al., 2010).

The presently reported case-control study aimed to assess the Hp gene polymorphism and ABO blood group relation with leprosy disease among leprosy patients from ALERT referral hospital in Ethiopia.

Statement of the problem

Study on leprosy is a subject of interest and essential in medicine, especially immunology. On the other way, although leprosy is rare in most developed countries, it is still a major public health problem in developing countries, where hundreds of thousands of new cases are diagnosed each year. In many of these countries, social stigmatization is an additional burden. Even if leprosy was identified as a major health problem in Ethiopia since 1950, still people with the disease face different stigmatization and socioeconomic problems. Therefore, it is important that research activities on leprosy should continue to reduce the disease burden and damaging impacts.

Additionally, a number of human genetic polymorphisms occur at high frequencies in leprosy endemic regions. Genetic variation, like haptoglobin genotypes, has an effect on oxidative stress, clearance of red cells, helper T cell balance, and recognition of pathogen antigens by the immune system. Several studies have shown the innate immune regulatory activities of haptoglobin to influence host's immune system which is associated with protection from clinical diseases. Accumulation of knowledge and a good understanding about the effect of this gene polymorphism is important in the study of leprosy immunology, for the development of acquired immunity and efforts to eradicate leprosy from the globe, and for other scientific values and social benefits. To date there is no study that has linked haptoglobin polymorphisms and ABO blood group to leprosy susceptibility in Ethiopia. Therefore, the purpose of this study was to assess the association of haptoglobin gene and ABO blood group among leprosy patients in Ethiopia.

2. Literature review

2.1. Haptoglobin

2.1.1. Haptoglobin protein

Haptoglobin, an acute-phase hemoglobin binding glycoprotein, is found in the plasma of humans and other mammals (Wicher and Fries, 2006). Principally, Hp is synthesized in the liver by hepatocytes, however, other non-hepatic cells or tissues such as airway epithelial cells of lung (Yang et al., 2000), leukocytes, fibrocytes, adipocytes, skin, spleen, kidney, arteries, and adipose tissue can also produce haptoglobin (Sadrzadeh and Bozorgmehr, 2004; Yang et al., 2000).

Human haptoglobin consists of four chains: two light α-chains and two heavy β-chains (Mircheva, 2010). The cells synthesize it as a single chain and then cleave into amino-terminal α-chain (light chain) and carboxyl-terminal β-chain (heavy chain) (Wejman et al., 1984). Whilst this post-translational processing, each α-chain binds to one β-chain and to other α-chains by disulfide bonds (Polticelli et al., 2008). The β-chain has 245 amino acids sequence and it does not show variation in all individuals. However, the α-chain exists in two major subunits (the α1-chain and the α2-chain) which are responsible for the presence of human haptoglobin polymorphism (Jue et al., 1983; Sadrzadeh and Bozorgmehr, 2004). The α1 subunit is made of 83 amino acids residue which weights approximately 9kDa containing a single cysteine residue (Lai et al., 2007). Whereas, the α2 subunit consists of 143 amino acids residue having 16kDa molecular weight and extra cysteine residue which is capable of forming multiple disulfide bonds (Lai et al., 2007; Sadrzadeh and Bozorgmehr, 2004). The α1 subunit is further divided into α1F (F, fast) and α1S (S, slow) chains. These two subunits of α1have different electrophoretic mobility due to the difference of amino acids at position 52 and 53 (Dobryszycka, 1997). Lysine in α1F is substituted by glutamic acid in α1S at position 53; likewise, aspartic acid in α1F is replaced by asparagines in α1S at position 52. This charge difference in amino acids constitution makes α1F to migrate faster than α1S during electrophoresis in acidic starch gel electrophoresis (Carter and Worwood, 2007; Wobeto et al., 2008).

Table 1: differences between the α1 variants, adapted from (Carter and Worwood, 2007)

	Hp1F	Hp1S
Amino acid 47	Valine	Valine
Amino acid 51	Asparagine	Asparagine
Amino acid 52	Aspartic acid	Asparagine
Amino acid 53	Lysine	Glutamic acid

Following its secretion, human haptoglobin circulates in plasma as a dimer (86kDa), or as a linear polymer (86 to 300kDa), or as a cyclic polymer (170 to 900kDa) (Sadrzadeh and Bozorgmehr, 2004).

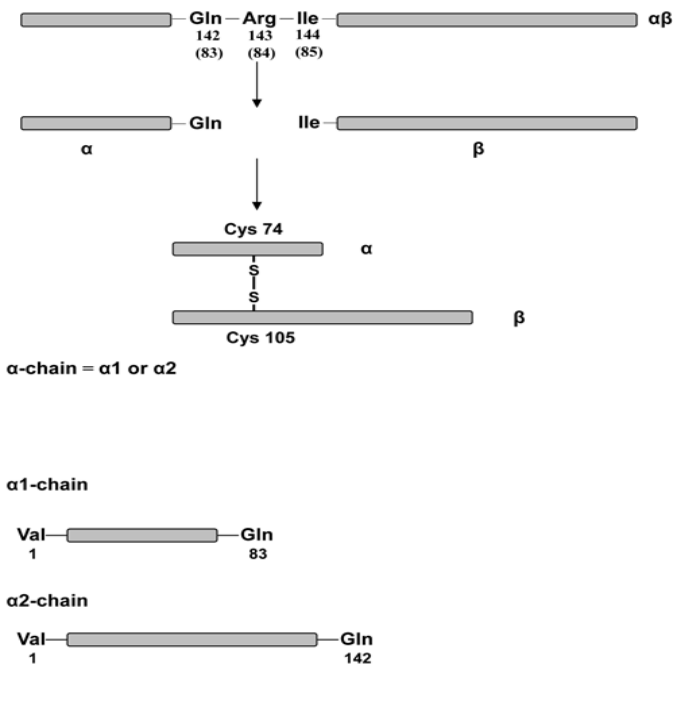


Figure 1: Post-translational cleavage of human Hp protein (Lai et al., 2007)

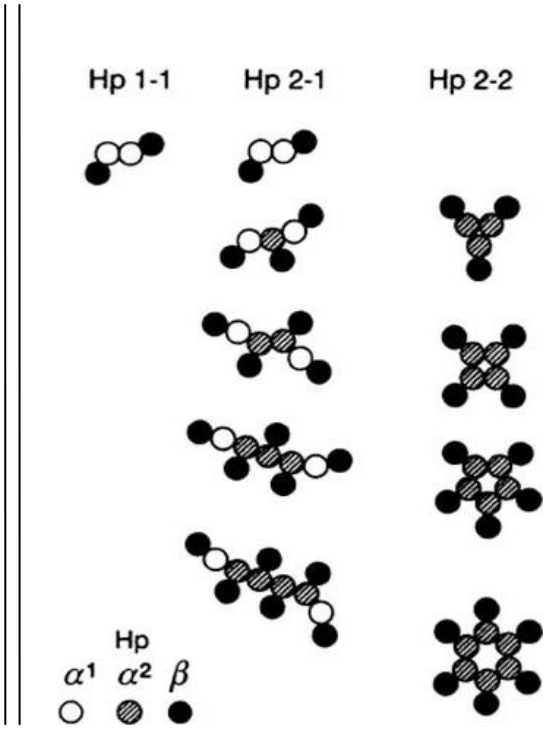


Figure 2: Structural arrangement of HP genotypes (Langlois and Delanghe, 1996a) Hp1-1, Hp2-1, and Hp2-2 are genotypes

2.1.2. Blood Haptoglobin concentration

Haptoglobin present in normal human serum at concentrations of between 0.3 and 2mg/ml (Dobryszycka, 1997). Its half-life in the free state is 3.5 days but the Hp-Hb complex has 10 minutes of half-life (Sadrzadeh and Bozorgmehr, 2004). The circulating amount of plasma haptoglobin shows great individual differences because of several factors such as: lack of Hp gene or allele, Hp allele type, health conditions, age, sex, hemolytic conditions, and other factors (Dobryszycka, 1997; Wobeto et al., 2008).

There are slight plasma Haptoglobin concentration differences between the three common phenotypes of Hp (Imrie et al., 2006). In normal individuals, the plasma concentration of Hp1-1 is much high compared to the other phenotypes. In contrast, the plasma concentration of Hp2-2 is very low relative to Hp11 and Hp12 (Fowkes et al., 2006; Langlois and Delanghe, 1996b). The concentration of Hp11 ranges between 0.57g/L and 2.27g/L, Hp12 is 0.44g/L to 1.83g/L and Hp22 is 0.38g/L to 1g/L (Uyoga, 2009).

In some rare cases, an individual may lack Haptoglobin protein in serum or the serum may contain reduced amount of Hp (Koda et al., 1998). Such situation is referred to as haptoglobinemia or hypohaptoglobinemia respectively (Delanghe et al., 1998). This might be caused due to different factors that lead to depletion of Haptoglobin in the plasma. The factors may include excessive hemolysis, sickle cell anemia, autoimmune hemolysis, and other pathological intravascular hemolysis (Rougemont et al., 1980; Thomsen et al., 2013). haptoglobinemia or hypohaptoglobinemia may also be caused as a result of Hp gene deletion.

Significant difference of Haptoglobin level in plasma has been shown at different age levels and sex. The plasma Haptoglobin concentration of healthy infant is lower than in healthy adults due to difference in transcription rate. The concentration of Hp in plasma gradually increases until 20 years of age (Wobeto et al., 2008). In healthy adults, the Haptoglobin concentration varies between 0.3 to 3g/L in human plasma (Asleh and Levy, 2005; Langlois and Delanghe, 1996a).

The other factors that cause concentration difference are inflammation, infection, tissue destructions, and malignant diseases. Haptoglobin concentration elevates in human by 3 to 8 folds in the presence of inflammation, bacterial infection, chemical irritant and connective tissue diseases (Dobryszycka, 1997; Imrie et al., 2004). Several studies indicated

Haptoglobin concentration elevation during infections and inflammations, tissue destruction, and various malignant diseases, including lung and bladder cancers (Benkmann et al., 1987), leukemia (Mitchell et al., 1988), breast cancer, malignant lymphoma, urogenital tumors, and esophageal squamous cell carcinoma (An et al., 2004). A study by Pan and colleague in patients with abdominal aortic aneurysm suggested the elevation of Hp plasma concentration in the patient with this disease, particularly with that Hp2-2 phenotype (Pan et al., 2011).

2.1.3. Haptoglobin gene

Haptoglobin protein is encoded by a gene located on chromosome 16q22.1 consisting of two common autosomal co-dominant alleles [Hp1 and Hp2] which code for different α-chains but identical β-chains (Maeda and Smithies, 1986). Since Hp2 is the result of breakage and reunion event at non-homologous positions within the fourth and second introns of two Hp1 subtype alleles (Maeda and Smithies, 1986) it contains two regions of similar nucleotide sequences which are approximately 1700bp length (Koch et al., 2002). Thus, Hp2 allele is 1.7kb longer than Hp1 allele.

The expression of Hp gene is induced by interleukin-6 (IL-6) (Oliviero and Riccardo, 1989) and three major possible Hp phenotypes are determined by these alleles; Hp1-1 which characterized by the smallest Haptoglobin dimmers [2($\alpha_1\beta$)]in the plasma and Hp1-2 which characterized by $\alpha_1\beta$ dimer and multiple $\alpha_2\beta$ units [($\alpha_1\beta$) + n($\alpha_2\beta$): n=0, 1, 2….] in the serum (Black and Dixon, 1968). Thirdly, Hp2-2 phenotype which differ from the others by multiple $\alpha_2\beta$ units having many disulphide bonds [n($\alpha_2\beta$): n=3, 4, 5…] (Dobryszycka, 1997; Lai et al., 2007).

The Hp1 allele consists of 5 exons which code for α1 and β polypeptides. The first four exons encode for α1-subunit having a length of 42 amino acids but the last (the 5^{th}) exon encodes for β-subunit polypeptide(Carter and Worwood, 2007). The α1 shows single nucleotide polymorphism in the 4^{th} exon which is located in codon 52 and 53 and produce the two sub-units of α1 [α1S and α1F] (Carter and Worwood, 2007).

Whereas, Hp2 has 7 exons and the first six exons encode for a larger polypeptide [α2-subunit]. The 7^{th} exon of this allele encodes for β-subunit polypeptide. DNA sequencing showed that this allele originates from the internal duplication of exon-3 and 4 of Hp1 and

then produces exon 5 and 6 of Hp2 respectively (Lai et al., 2007; Maeda and Smithies, 1986). The intragenic duplication within the human *Hp2* allele was formed by a non-homologous [probably random] crossing-over within different introns of two Hp^{1} alleles [probably in an Hp^{1F} /Hp^{1S} heterozygote] (Maeda and Smithies, 1986).

Deletion in Hp gene results in absence of Haptoglobin expression (Koda et al., 1998). This condition causes ahaptoglobinemia or hypohaptoglobinemia which characterized by lack or low concentration of Haptoglobin in plasma respectively. Individuals with such feature phonotypicaly could be either Hp0-0 or Hp1-0 or Hp2-0 (Koda et al., 1998). Homozygous individuals for Hp0 have no Haptoglobin in their serum whilst those heterozygous for this allele have low concentration of the protein in their serum. This genetically determinant of ahaptoglobinema or hypohaptoglobinema is as a result of ~28kbp spanning deletion from the upstream region of exon-1 of the Hp gene to intron-4 of Haptoglobin related gene (Koda et al., 2000).

Haptoglobin related gene (Hpr) is located on chromosome-16 approximately 2.2kb downstream from the Hp gene on the 3' side (Maeda and Smithies, 1986; Nielsen and Moestrup, 2009). Hpr gene shares high degree [>90%] of nucleotide sequence homology with Hp gene and thought to be originated from the duplication of Hp (Maeda and Smithies, 1986). The first intron of Hpr has a feature of retrovirus-like element which is 9kbp longer than that of equivalent intron of Hp. Similarly, there is ATTAT sequence on both sides of retrovirus-like sequence and only one in Hp gene at equivalent position (Maeda and Smithies, 1986; Maeda et al., 1986). Hpr gene codes for Hp related (Hpr) functional plasma protein whose amino acid sequence differs about by 8% from that of the Hp1F allele (Maeda and Smithies, 1986).The Hpr protein has been identified in normal human serum at 5-10% of the Hp levels (Carter and Worwood, 2007). This protein binds with Hb low affinity but the Hpr-Hb complex is not recognized by the CD163 receptor. Hp-related protein form trypanosome lyses factor when associated with Hb as a result the ingestion of Hpr-Hb complex by trypanosome causes iron toxicity and the trypanosome autodigestion through lysosomal membrane lyses (Langlois and Delanghe, 1996a).

2.1.4. Geographic distribution of Hp allele

The frequency of the HP1 and HP2 alleles varies in the worldwide human population depending on the racial origin (Wobeto et al., 2008). For instance, the frequency of HP1 allele varies from about 0.07 in parts of India to over 0.7 in parts of West Africa and South America (Carter and Worwood, 2007). In Europe, the allelic frequency of Hp1 is about 0.43 and Hp2 allele exists in frequency of 0.57. In America the approximate allele frequency of Hp1 is 0.54 and 0.46 for Hp2 and the native populations in South America have the highest frequency of the Hp1 allele [0.7]. Similarly, in West Africa and East Africa the frequency of Hp1 allele is dominant (Quaye, 2008). On the other hand, Asian populations have the lowest frequency of the Hp1 allele [0.2] (Wobeto et al., 2008). The frequency of Hp allele in different population of some country around the world is summarized in the following Table2.

Table 2: Hp1 allele frequencies in different populations of the world (Wobeto et al., 2008)

Continent	Country	Population studied	n	Hp1 allele frequency
Africa	Central Africa Republic	Pygmies	919	0.35
	Ghana	General population	123	0.52
	Kenya	Luo tribe (north of Lake Victoria)	227	0.57
	Sudan	General population	208	0.54
	Ethiopia	???	???	???
America	Brazil	Pacaás Novos Indians Yanomama Indians (Venezuelan border) Içana River Indians (northern Amazon region)	222 984 133	0.82 0.83 0.43
	Canada	Mixed(French Canadians, Africans, Asians and others)	358	0.39
	USA	Indian (Alabama Coushatta) General population	143 3273	0.37 0.39
	India	Punjab (northern India) - Jat Sikh group	192	0.23

Asia	Israel	General population	757	0.33
	China	General population	667	0.33
Europe	Norway	Oslo	6668	0.38
	Germany	Hamburg (northern Germany)	1725	0.39
	Belgium	General population	918	0.40

2.1.5. Biological function of haptoglobin

2.1.5.1. Oxidative stress prevention

Hemoglobin is iron containing oxygen transporting metallo-protein in the red blood cells. Therefore, it is crucial for life, however, accumulation of free plasma Hb is highly toxic to cells because of the following two reasons (Boretti et al., 2009). 1) Its heme group is lipophilic and can readily intercalate into cell membranes to disrupt the lipid bilayers. 2) The iron component of Hb promotes the accumulation of cell-damaging oxygen radicals (reactive oxygen species, ROS) and lipid peroxides by means of the Fenton ($H_2O_2 + Fe^{2+} > Fe^{3+} + OH^-$ + OH) and Haber-Weiss reactions (Langlois and Delanghe, 1996b; Quaye, 2008). These might lead to oxidative stress which is an imbalance between the systemic manifestation of ROS and a biological system ability to readily detoxify the reactive intermediates or to repair the resulting damage. The disturbance of normal redox sate of cells causes toxic effects that damage all components of the cell including proteins, DNA, and lipids (Schafer and Buettner, 2001).

Hemoglobin is released into the circulation by intravascular hemolysis and /or extra-vascular hemolysis or normal red blood cell (RBC) turnover. In most cases, intra-vascular hemolysis become a severe pathological complication when it is accelerated by various conditions including autoimmune situations, infectious, and inherited disorders (Wagener et al., 2003).

In plasma, free hemoglobin binds Haptoglobin with extremely high affinity (KD ~10^{-15} M) in a ratio of 1Hp:1Hb (Mircheva, 2010; Saeed et al., 1981). The haptoglobin-hemoglobin complexes are rapidly removed and degraded by macrophages through CD163 mediated endocytosis. CD163 is cell-surface receptor which is expressed on the surface of mature macrophages and monocytes (Kristiansen et al., 2001). The expression of CD163 is up-regulated by different anti-inflammatory cytokines such as glucocorticoids, interleukin-6, and

interleukin-10 and it can be down-regulated by pro-inflammatory molecules like IL-4, interferon-γ, TNF-α, and lipo-polysaccharide [LPS] (Sadrzadeh and Bozorgmehr, 2004; Thomsen et al., 2013).

Following the association of Hp-Hb-CD163, the Hp-Hb complexes are internalized by macrophages and the globin moieties are degraded in the lysosome. Whereas, the heme fraction is metabolized by heme oxygenase-1 [HO-1] and converted into bilirubin, carbon monoxide, and iron (Thomsen et al., 2013). The HO-1catalized products are important anti-oxidative and anti-inflammatory metabolites which maintain cellular homeostasis. For example, carbon-monoxide (CO) appears to be involved in the inhibition of platelet aggregation (Wunder and Potter, 2003) or in the induction of the expression of anti-inflammatory cytokine IL-10 and inhibition the expression of pro-inflammatory cytokines such as TNF-α and IL-1β (Thomsen et al., 2013). In the same way, biliverdin is reduced into bilirubin by biliverdin redactase enzyme and involves in the anti-oxidative activities (Wunder and Potter, 2003). Likewise, Fe^{2+} is stored as ferritin [the primary storage of iron] or it is transported back to bone marrow via plasma transferrin for the synthesis of new hemoglobin.

The chelation of Fe^{2+} by ferritin inhibits Fe^{2+}catalyzed generation of reactive oxygen species via the Fenton reaction. This activity provides protection against oxidative stress and at the same time prevents the loss of iron through kidneys and protects kidneys [renal tissue] from oxidative damage caused by hemoglobin (De Kleijn et al., 2002; Quaye, 2008). Similarly, it provides non-specific defense against pathogenic bacteria by preventing iron utilization for their growth (Langlois and Delanghe, 1996b).

The binding affinity of Hp-Hb complex with CD163 receptor and Hb clearance vary based on Hp phenotype. Hp 1-1 individuals have greater hemoglobin binding capacity compared to individuals with Hp1-2 and Hp2-2 phenotypes. Hp2-2-Hb complex has the least CD163 binding affinity (Nielsen and Moestrup, 2009) as a result, the clearance of Hb from circulation is less effective in Hp2-2 individuals. The reason of this is attributed to the larger molecular size of α2 subunit (Gueye et al., 2006; Langlois and Delanghe, 1996b). Therefore, Hp 1-1 phenotype is able to bind more hemoglobin on a Molar basis than the two Hp phenotypes. Similarly, Haptoglobin molecules in individuals with Hp1-1 phenotype are also more efficient antioxidants, since the smaller size of haptoglobin 1-1 facilitates its entry to

extravascular sites of oxidative tissue injury compared to products of the Hp2 allele (Melamed-Frank et al., 2001). In another way, there is a significantly greater glomerular sieving of Haptoglobin in subjects with Hp-1-1 phenotype.

2.1.5.2. Antioxidant activity

Compromised oxygen supply to cells, which occurs during infection, injury, and heart or lung problems, triggers the generation of reactive oxygen species such as hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and free radicals including the hydroxyl radicals (-OH) and the superoxide anion (O_2^-) (Valko et al., 2007). The generation of these molecules causes damage to cell membrane, proteins, lipids, and DNA (Schafer and Buettner, 2001).

Similarly, cells of the immune system generating ROS also produce reactive nitrogen species which are equally damage cell membrane and DNA (Quaye, 2008). The extravascular anti-oxidant function of Haptoglobin involves in maintenance of reverse cholesterol transport, inhibition of cyclooxygenase (COX) and lipooxygenase (LOX), activation of neutrophils, and other antioxidant activities (Quaye, 2008). This antioxidant property of Haptoglobin protects low density lipoproteins from Cu^{2+} induced oxidation (Tseng et al., 2004).

Anti-oxidants are molecules that inhibit the oxidation of other molecules. Cellular non-enzymatic antioxidants that participate in oxidative stress defense include: ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E), glutathione (GSH), carotenoids, and flavinoids (Khansari et al., 2009). Tseng et al. showed that Haptoglobin possesses an antioxidant activity that exceeds ascorbic acid (vitamin C) and contributes significantly to this process, particularly at extravascular sites (Tseng et al., 2004).

The oxidative protection of Hp1 is more effective compared to Hp2 allele (Moreira et al., 2009) thus, the anti-oxidant activity of Hp is phenotype dependent. Hp1-1 is the most active antioxidant phenotype and Hp2-2 has the least active antioxidant property (Melamed-Frank et al., 2001), with Hp2-1 being intermediate phenotype (Gueye et al., 2006).

2.1.5.3. Hp and nitric oxides

Nitric oxide is highly reactive short-lived free radical gas which is produced and released by vascular endothelial cells to promote smooth muscle relaxation and phagocytes [monocytes, macrophages and neutrophils] as part of immune system. It contributes to vascular

homeostasis by regulating vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium (Bredt, 1999). The nitric oxide secreted as an inflammation and immune response is important as a toxic defense molecule against infectious organisms. It also regulates the functional activity, growth, and death of many immune and inflammatory cell types that results in an anti-inflammatory effect. Similarly, NO also involves in the modulation of neurotransmitter function in the central and peripheral nervous systems(Wobeto et al., 2008).

Free plasma hemoglobin released by hemolysis scavenges nitric oxide via deoxygenation. The consumption of nitric oxide by Hb impairs NO signaling activity and then reduces its toxic defense against infectious organisms (Thomsen et al., 2013). Furthermore, it minimizes the regulatory functions and the anti-inflammatory efficiency of NO (Tripathi, 2007). However, the binding of Haptoglobin to hemoglobin limits hemoglobin interaction with nitric oxide and thus maintaining NO activities (Sadrzadeh and Bozorgmehr, 2004). Thus, NO scavenging rate depends on the rate of Hb clearance, and therefore, on the Hp phenotype. Hp1-Hb complexes are cleared faster than Hp2-Hb complexes (Azarov et al., 2008).

2.1.5.4. Immunomodulation

Hp regulates the balance of T-helper cells [Th1and Th2]. Helper T-cell type 1 (Th1) and helper T-cell type 2 (Th2) are the two groups of T lymphocytes that regulate cellular and humoral immune responses (Sadrzadeh and Bozorgmehr, 2004).

Th1 cells are involved in the cellular [phagocytes and dendritic cells] immune response and host defense against intracellular pathogens (Kidd, 2003). These cells are characterized by the production of pro-inflammatory cytokines like IFN-γ, IL-2, and lymphotoxin-α (LTα). Th1 cells are centrally involved in cell-mediated immunity. The cytokines produced by Th1 cells stimulate the phagocytosis and destruction of microbial pathogens by macrophages and other lymphocytes. Several chronic inflammatory diseases have been described as Th1-dominant diseases.

Th2 cells are involved in the humoral [cytokines, complement, acute phase proteins and leukotrienes] immune response and host defense against extracellular parasites (Kidd, 2003). These cells are characterized by the production of IL-4, IL-5, IL-6, IL-10, and IL-13. Th2 cells are thought to play a role in allergic responses. Cytokines like IL-4 generally stimulate

the production of antibodies directed toward large extracellular parasites, while IL-5 stimulates eosinophil response toward large extracellular parasites. Allergy and atrophy are thought to be Th2-dominant conditions.

When Th1 cells produce IFN-γ, this prompts macrophages to produce TNF and toxic forms of oxygen which destroy the microorganisms within the phagosomes and lysosomes. On the other hand, when Th2 cells produce IL-4 and IL-10, these cytokines block the microbial killing that is activated by IFN-γ. Th1-Th2 imbalance is responsible for the susceptibility of various pathologic conditions such as parasitic and other infections, allergies, and autoimmune disorders (Quaye, 2008; Sadrzadeh and Bozorgmehr, 2004).

Hp regulates the balance of Th1and Th2 by cytokine production from macrophages exposed to Hp-Hb complex via CD163 dependent mechanism (Guetta et al., 2007; Wobeto et al., 2008). For example, the Hp1-1–Hb complex promotes the secretion of more IL-6 and IL-10 than the Hp2-2–Hb complex (Guetta et al., 2007) and hence, increases Th2 dependent immune response.

Haptoglobin modulates the immune response by binding to the membrane proteins of many different immunologic cells including monocytes, granulocytes, and natural killer cells (Berkova et al., 1999). These receptors are integrin family receptors [MAC-1 (CD11b/CD18)] which are expressed on dendritic cells, neutrophils, monocytes, macrophages, NK cells, and a small subset of T cells (El Ghmati et al., 1996). In the same way, Hp is also ligand for CD22 lectin on B-lymphocytic cells that mediates B-cell interactions with other cells (Langlois et al., 1997). Interaction between these cell surface proteins and Hp has been associated with changes in cell proliferation, expression of stimulated cytokine genes, and cell motility (Huntoon et al., 2008). Neutrophils possess specific binding site for haptoglobin and by binding to these cells Hp blocks the response of neutrophils to a variety of agonists with defined plasma membrane receptors (Oh et al., 1990). Hence, Hp prevents neutrophils from respiratory burst activity (superoxide production) and the intracellular rise of calcium. To a minor extent, Hp can also be ligand for CD4+ and CD8+. Experimental models of colitis showed that Hp has a protective role in inflammatory colitis, most likely by inhibiting the production of Th1 and Th17 cytokines (Ma´rquez et al., 2011).

In general, Haptoglobin involves in the modulation of several cellular activities like; prostaglandin synthesis, leukocyte activation [activation of innate and adaptive immune responses], modulation of cytokine patterns, and tissue repair and regeneration processes. Furthermore, Hp inhibits or enhances proliferation of B-cells in response to bacterial endotoxins, depending on its concentration.

2.1.5.5. Hp and inflammation

Inflammation is one of the first responses of the immune system to infection (Kawai and Akira, 2006). Inflammation is produced by eicosanoids and cytokines, which are released by injured or infected cells. Eicosanoids include prostaglandins that produce fever and the dilation of blood vessels associated with inflammation, and leukotrienes that attract certain white blood cells [leukocytes] (Miller, 2006). Common cytokines include interleukins that are responsible for communication between white blood cells; chemokines that promote chemotaxis; and interferons that have anti-viral effects. Growth factors and cytotoxic factors may also be released. These cytokines and other chemicals recruit immune cells to the site of infection and promote healing of any damaged tissue following the removal of pathogens (Martin and Leibovich, 2005).

Hp exerts an anti-inflammatory action by inhibiting inflammatory mediators (Opal and Vera, 2000). The level of Hp increases at the sites of infection or injury to modulate these acute inflammatory responses (Berkova et al., 1999). Prostaglandin is pro-inflammatory substance which derived from the metabolism of arachidonic acid in inflammatory cells and induces the expression of inflammatory cytokines. Haptoglobin serve as anti-inflammatory agent by potentially inhibiting the enzyme prostaglandin synthase which involves in prostaglandin biosynthesis during inflammation (Jue et al., 1983; Saeed et al., 1981). Consequently, it maintains the balance of pro-inflammatory and anti-inflammatory processes. Most importantly, the prostaglandin inhibitory effect of Hp11 is more compared to Hp22 and Hp12 has medium efficiency (Langlois and Delanghe, 1996b; Saeed et al., 2007). Haptoglobin also exhibits a marked inhibitory activity of cathepsin (Pagano et al., 1980; Snellman and Sylven, 1967). In addition to its interaction with immune cells [leukocytes], Hp involves in inflammatory reactions like respiratory inflammatory diseases (Yang et al., 2000).

In addition to its antioxidant role, Hp mediated degradation of Hb produces metabolites that actively participate in anti-inflammatory processes (Thomsen et al., 2013). For instance, the carbon monoxide (CO) derived from the metabolism of heme by HO-1 has been shown to be involved in the regulation of intestinal inflammation (Takagi et al., 2010). Furthermore, both in-vivo and in-vitro studies show carbon monoxide at low concentrations differentially and selectively inhibited the expression of lipopolysaccharide-induced pro-inflammatory cytokines tumor necrosis factor-α, interleukin-1β, and macrophage inflammatory protein-1β and increased the lipopolysaccharide-induced expression of the anti-inflammatory cytokine interleukin-10 (Otterbein et al., 2000). Similarly, bilirubin is also a good anti-inflammatory metabolite [product] of the Heme oxygenase-1 (HO-1). As a result, nowadays, carbon monoxide and bilirubidin (biliverdin) can be part of therapeutic strategy for treatment of various inflammatory illnesses (Otero Regino et al., 2009).

On the other hand, the activation of the CD 163 also induces a signal mediated by protein tyrosine kinase, leading to the secretion of anti-inflammatory cytokines and giving rise to a connection between the clearance function of the Hp and their immune modulatory functions. The binding of the Hp–Hb complex to CD163 induces intra-cellular signaling leading to increased heme oxygenease activity and release of anti-inflammatory cytokines (IL-10) (Opal and Vera, 2000).

2.1.6. Hp polymorphism and disease association

2.1.6.1. Hp and infectious diseases

Haptoglobin gene polymorphism has been associated with the prevalence of several inflammatory diseases, including atherosclerosis, autoimmune disorders, and other several infectious diseases (Langlois and Delanghe, 1996b) such as malaria, tuberculosis, AIDS, hepatitis C and other (Sadrzadeh and Bozorgmehr, 2004). In lungs, haptoglobin is synthesized locally and is a major source of antimicrobial activities in the mucous layer and alveolar fluid and also has an important role in protecting against infection (Yang et al., 2000).

Several studies indicated the role of *Hp1* and *Hp2* alleles in susceptibility or protection against infections and their selective advantage in various populations. Individuals with Hp 2-2 are more susceptible to develop tuberculosis and have a higher risk of mortality of

tuberculosis (Sadrzadeh and Bozorgmehr, 2004). Patients infected with HIV-1 and possess Hp2-2 phenotype have a worse prognosis which related to a more rapid rate of viral replication (Langlois and Delanghe, 1996b), and have lower survival rate relative to the Hp1-1 phenotype. In these individuals [HIV-1 patients with Hp2-2] plasma HIV-1 RNA concentrations are higher than in those with other Haptoglobin phenotypes (Sadrzadeh and Bozorgmehr, 2004). In patients with hepatitis C, Hp 1-1 is overrepresented, and the Haptoglobin phenotype affects the immune response after hepatitis B vaccination. Hp2-2 individuals develop lower levels of antibodies after vaccination against hepatitis B than those with Hp1-1 or Hp2-1 phenotypes (Sadrzadeh and Bozorgmehr, 2004). People with Hp 2-2 have a stronger antibody response to typhus and tetanus vaccination. A study from Gambia also indicated that genetic variation in Hp affects susceptibility to active trachoma. Table 3 summarizes the relationship between some infectious diseases and Hp phenotype/genotype.

Table 3: Association between human Haptoglobin genotypes and infectious diseases (Wobeto et al., 2008).

Infectious disease	**Population studied**	**Conclusion**
American trypanosomiasis	80 patients from southeastern Brazil (Caucasian and Afro-descendants) and 197 controls	Hp2-2 phenotype much more frequent in patients with any form of American trypanosomiasis, in patients with the indeterminate form of the disease and in patients with the chronic combined form
Filariasis	605 patients from northern Brazil and 597 controls	No association between disease and HP phenotypes
AIDS	653 HIV-infected Caucasian patients (493 from Belgium and 160 from Luxembourg), and 204 controls	Higher mortality for the Hp2-2 group; median survival time of 11.0 years (Hp 1-1 and Hp 2-1) versus 7.33 years (Hp 2-2). Plasma HIV-1 RNA levels prior to antiviral therapy and their increase over one

		year was highest in Hp2-2 patients. Hp2-2 was associated with higher serum iron, transferrin saturation and ferritin levels, and low vitamin C concentrations
AIDS	387 patients from southeastern Brazil (Caucasians and Afro-descendants) and 142 controls	No association between Hp genotypes and either HIV status or indices of HIV progression
Tuberculosis	84 Russian patients	Hp2-2 patients had large cavities of tissue destruction and more advanced dissemination
Tuberculosis	98 African patients from Zimbabwe and 98 controls	Equal susceptibility to clinical pulmonary tuberculosis disease among different Hp phenotypes. Hp2-2 patients had a higher risk of mortality
Nephorotic tuberculosis	152 Russian patients	Hp2-2 patients with more severe nephritic tuberculosis
Chronic hepatitis C	239 patients from Belgium and 220 controls	Hp phenotype distributions and HP allele frequencies in the patient group differed significantly from those in the reference population. There were no significant differences between Hp phenotype distribution and hepatitis C virus types or response to interferon alpha therapy
Malaria	273 Sudanese patients and 208 controls	Hp1-1 associated with susceptibility to falciparum malaria

		and development of severe complications
Malaria	113 *P. falciparum* malaria children from coastal Ghana and 42 controls	Hp1-1 phenotype associated with susceptibility to *P. falciparum* malaria in general, and to the development of severe disease in particular
Malaria	182 individuals from the Brazilian western Amazon (Amerindians, Caucasoids and Afro-descendants)	No association between Hp phenotypes and susceptibility to malaria infection was found
Malaria	119 Indigenous pregnant African women from western Cameroon	Hp1-1 phenotype may play a role in susceptibility to placental infection by *P. falciparum* during pregnancy. Hp1-1 women had higher parasite prevalence in peripheral blood and placentas. Significant difference in parasite density between Hp1-1 and Hp2-2 phenotypes for placental infection but not for maternal peripheral blood infection
Hansen's diseases (Leprosy)	1,009 patients from southern Brazil (935 Caucasians and 74 Afro-descendants), and controls (500 Caucasians and 381 Afro-descendants)	No significant relationships could be established either between Hp and the type of Hansen's disease or between Hp and severity of the disease

2.1.6.2. Hp and non-infectious diseases

Studies on haptoglobin have shown the susceptibility or resistance of individuals to many non-infectious diseases based on the Haptoglobin phenotype. Some of the noninfectious diseases associated with Hp include: acute myocardial infarction and atherosclerosis

individuals with Hp2-2 have an increased risk of atherosclerotic vascular disease and acute myocardial infarction (Moreno et al., 2008; Suleiman et al., 2005). Pregnancy-induced hypertension – associated with Hp2-2; diabetic mellitus - Hp 2-2 phenotype is more prevalent than other Haptoglobin genotypes (Asaf et al., 2009); in diabetic patients, the risk of cardiovascular disease is much greater in Hp 2-2 than in those with Hp 1-1 or Hp 2-1 (Levy et al., 2007); cancers – for example the prevalence of breast tumors has been found to be higher in women with the Hp1-1 phenotype (Wobeto et al., 2008).; neurological disorders – associated with Hp2-2 (Sadrzadeh and Bozorgmehr, 2004); and similarly many other diseases like: coronary heart disease, cerebrovascular disease, peripheral arterial disease, and inflammatory heart disease have been associated with Haptoglobin phenotype (Wobeto et al., 2008). A genetically determined T-lymphocyte mediated chronic inflammatory disorder called Celiac disease is related to Hp in which Hp1-2 is predominant genotype (Papp et al., 2008).

2.2. Leprosy

Leprosy is a human chronic infectious disease caused by aerobic rod-shaped bacteria called *Mycobacterium leprae* which is gram positive, acid fast bacilli, obligate intracellular organism, reproduces by binary fission, and invades skin and peripheral nerves. Leprosy causative agent, *Mycobacterium leprae,* was discovered by physician Gerhard Armauer Hansen in Norway in 1874 (Pinheiro et al., 2011; Walker and Lockwood, 2006). This discovery made it the first bacterium to be identified as causing infectious disease in humans. *M. leprae* grows and multiplies in humans, footpads of mice, armadillos and immune-suppressed rodents. *M. leprae* is the slowest growing bacterial pathogen which cannot be cultured in vitro [outside of the cell] (Barker, 2006).

M. leprae enter the body by way of the mucous membranes of the nose or mouth or through abrasions in the skin. And then, begin to multiply within the body of host and cause an impairment of nerve function and damages of limbs, skin, and eyes that lead to chronic disabilities (Walker and Lockwood, 2006). The bacteria infect and replicate in the intracellular vesicles of macrophages, endothelial cells, and the nonmyelinating and myelinating Schwann cells in the peripheral nerve which leads to axonal dysfunction and demyelination (Barker, 2006; Scollard, 2008).

Human beings are the main natural reservoir for *M. leprae* (Rees, 1969). Some animals other than humans are thought to be the source of *M. leprae* although armadillos are the only confirmed source. The mode of transmission is still unknown but it is believed to be that the bacteria spread from person to person primarily through inhalation of *M. leprae* that are excreted from the nasal passages of the untreated borderline lepromatous and polar lepromatous patients (Noordeen, 1994; Walker and Lockwood, 2006). However, there are few reports of skin to skin contact and insect bite as mode of transmission.

Other factors that may play a role in the causes of leprosy include genetic factors, the extent of exposure, and environmental conditions. The incidence of leprosy is highest in extremely poor countries of the globe. Therefore, environmental factors such as unhygienic living conditions, overpopulation, and malnutrition may also be contributing factors favoring the infection.

2.2.1. Diagnosis and classification of leprosy

Leprosy symptoms usually do not begin until more than one year after infection [usually appear within an average years of five to seven after infection]. Once the symptoms begin, they progress slowly and they are myriad and include anesthetic polymorphic skin lesions and peripheral neuropathy. The diagnosis of leprosy is mainly based on the clinical signs and the symptoms of the disease which is then confirmed by microscopic examination of biopsy specimens. In an endemic country or area, the following cardinal signs should make an individual suspect for leprosy (Walker and Lockwood, 2006):

- Hypopigmented or reddish patches with loss of sensation, with or without thickened of peripheral nerves and
- Positive skin smears.

Currently, two systems exist in the medical literature to classify leprosy patients: The Ridley–Jopling system (Ridley and Jopling, 1966) which uses clinical and histopathological features, and the bacteriological index. The different categories of the features are correlated with the activity of the host immune response. The second system is the WHO system which is a simple classification system and uses the number of skin lesions.

2.2.1.1. Ridley–Jopling system

The Ridly-Jopling system was proposed by Dennis S. Ridley and W H. Jopling in 1960 and published in 1966. The system uses clinical, histological and immunological criteria to classify leprosy patients across the spectrum (Ridley and Jopling, 1966). Bacillary load measured by bacilloscopic examinations (cutaneous biopsy and skin smear) and the cell-mediated immune response time, which is evaluated from the result of Mitsuda's intradermal test are used to categorize patients (Ridley and Jopling, 1966). The Ridley-Jopling classification links immune status and clinical manifestations. Based on these immunopathological criteria, patients are divided into six clinical categories that range from the mildest indeterminate form to the most severe lepromatous type. More severe forms arise because of less effective immune response to the infection.

Indeterminate leprosy [I]: is a very early form of leprosy which either be cured or progress to one of the other forms of leprosy depending on immune status. Patients with indeterminate leprosy are characterized by a few hypopigmented macules that can heal spontaneously and may persist or advance to other forms.

Tuberculoid leprosy [TT]: Tuberculoid leprosy shows a well developed cell mediated immune response which characterized by T-cell and macrophage activation with very few bacilli in the tissues. Patients with TT have a few hypopigmented macules, some are large and some become anesthetic (lose pain sensation); some neural involvement in which nerves become enlarged; spontaneous resolution in a few years, persists or advances to other forms.

Borderline tuberculoid leprosy [BT]: BT is similar to TT except that the lesions are smaller and more numerous with less nerve enlargement. BT may persist, or convert back to tuberculoid form, or advance to other forms.

Borderline leprosy [BB]: the main features that characterize BB are numerous reddish and asymmetric plaques along with moderately anesthetic regional adenopathy (swollen lymph nodes). Borderline leprosy may persist or improve or worsen.

Borderline lepromatous leprosy [BL]: borderline lepromatous leprosy is immunologically unstable, and the disease manifestations may shift toward either pole. BL is characterized by numerous skin lesions of all kinds: macules (flat lesions),

papules (raised bumps), plaques, and nodules. Sometimes there may or may not with anesthesia. This form of leprosy may persist, or regress, or progress to lepromatous leprosy.

Lepromatous leprosy [LL]: Lepromatous leprosy is characterized by the absence of macrophage activation and high number of bacilli. Patients with LL have no cell mediated immunity. Early lesions are pale macules (flat areas) that are diffuse and symmetric; later many *M. leprae* organisms can be found in them. As the disease progresses, nerve involvement leads to anesthetic areas and limb weakness; progression leads to aseptic necrosis (tissue death from lack of blood to area), lepromas (skin nodules), and disfigurement of many areas including the face. The lepromatous leprosy form does not go back to the other less severe forms.

2.2.1.2. World Health Organization (WHO) system

According to WHO system, patients are classified on the number of skin and nerve lesions and skin smear positivity/negativity. The number of lesion is less than or equal to five for paucibacillary (PB) and greater than five for the multibacillary (MB) form and if skin smear is positive despite the number of skin lesion it is considered as multibacillary. In relation to Ridley-Jopling system, paucibacillary includes TT and BT whereas multibacillary contains BB, BL, and LL.

2.2.2. Immunology of leprosy

Host defense mechanisms consist of innate immunity which provides initial protection against pathogen and adaptive immunity which mediate the later protection against microbes. The adaptive immune system is grouped into two branches – humoral immunity and cell-mediated immunity. In the former, the protective function of immunization could be found in the humor (cell-free bodily fluid or serum) and antibody molecules play a central role during immune response. In the later, the protective function of immunization is associated with cells and, therefore, it is a part of the immune response that does not involve antibodies rather involves in the actions of macrophages, natural killer (NK) cells, cytotoxic T cells (CD8+ cells), and the actions of various cytokines. Although these two streams of immune response play a key role during *M. leprae* infection, cell mediated immunity is extremely important

during the infectious process of *M. leprae* due to the inability of B-cells to mount a useful immune response to pathogen.

Tuberculoid leprosy (TT) patients have low humoral immune response and strong cell mediated immunity with a large Th1-dominant T-cell cytokine response (Walker and Lockwood, 2006) which may cause collateral damage to tissues by granulomatous inflammation (Scollard et al., 2006). TT patients have well-formed granulomas at the sites of lesion with low intracellular bacilli load and delayed hypersensitivity against *M. leprae* (Spierings et al., 2000). In contrast, lepromatous leprosy (LL) patients are characterized by low cell-mediated immune response and high Th2- dominant cytokines that promote humoral immune response (Spierings et al., 2000). LL patients have poorly formed or absent granulomas with abundant bacilli. Furthermore, lepromatous leprosy patients show *M. leprae* specific cellular non-responsiveness and fail to clear the bacteria. The borderline leprosy [BT, BB, and BL] patients show unstable immune response with intermediate intracellular bacillary loads and cellular immunity *to M. leprae* and its antigen (Walker and Lockwood, 2006).

Reversal reaction [type-1 reaction] occurs in patients with unstable immune response against bacilli [BL, BB, or BT] or TT leprosy patients. RR is characterized by rapid shift from Th1-dominant T-cell response to Th2-dominant response and tissue damage. Erythema nodosum leprosum [ENL, type2 reaction] is classified towards the lepromatous pole [LL, BL, BB] and usually affects patients showing Th2 response and high bacilli loads. ENL is characterized by increased serum level of TNF-α and immune complex–associated vasculitis, panniculitis, and uveitis.

2.2.2.1. Cytokines

Cytokines are large group of glycoproteins which are produced by immune or non-immune cells. They include interferons (INF), nterleukins (IL), and various colony-stimulating factors (CSFs) (Kidd, 2003). Cytokines act as molecular signals for communication between cells of the immune system (Madan et al., 2011).

Th1 and Th2 mediated immune response are thought to be regulated by a balance between type 1 cytokines, including IL-2, IFN-γ, TNF-α, IL-12, and the type 2 cytokines, such as IL-

4, IL-6, and IL-10 (Stefani et al., 2003). IL-10 cytokine is also produced by monocytes and activated T-cells (Jarduli et al., 2013). IL-4 and IL-10 inhibit the secretion of IL-12 and IFNγ thereby blocking IL-12 induced Th1 differentiation and IFNγ release from Th1 and NK cells (Stefani et al., 2003) and then affect the immune-modulatory effect of IL-12. Furthermore, many studies also indicated that IL-10 suppresses the production of inflammatory mediators during bacterial infection and aids the development of Th2 immunity (Jarduli et al., 2013). IL-12 induces Th1 proliferation and serves as pro-inflammatory cytokine by promoting the production of IFN-γ and by enhancing the proliferation and cytotoxicity of NK and T-cells (Jarduli et al., 2013; Modlin, 1994). IFN-γ activates macrophages and plays a key role in antimycobacterial Th1 mediated immune response. Likewise, IFN-γ activates antimicrobial mechanisms in macrophages by inducing Nitric oxide synthase enzyme which catalyze the production of Nitric oxide [a powerful microbicidal molecule] (Madan et al., 2011). TNF-α accomplishes many Th1 mediated pro-inflammatory activities which include macrophage activation during infection.

Studies showed the predominance of IL-2, TNF-α and IFN-γ mRNA transcripts in the TT lesion and IL-5, IL-4, and IL-10 in LL, consistent with Th1 and Th2 patterns, respectively (Modlin, 1994). Patients with lepromatous leprosy (LL) but not borderline tuberculoid leprosy (BT) have defective cell-mediated immune responses to *M. leprae* (Mohagheghpour et al., 1985). Additionally, resent analysis of serum level of cytokines in leprosy patients indicated significant rise of IL-10 and IL-1β cytokines in multibacillary patients (Madan et al., 2011). again another study also indicated correlation between IL-6 and type 2 leprosy reaction (Jarduli et al., 2013).

2.2.2.2. Th1/Th2 cellular role

T-helper cells (CD4+ T-cells) are special subpopulation of T-cells that provide protection against different pathogens. CD4+ functions by activating B-cells to produce antibodies and by regulating the response of other immune cells (Tsai and Yu, 2014). T-helper cells secrete lymphokines that stimulate cytotoxic T cells and B cells to grow and divide as well as recruit neutrophils, eosinophils, basophils to the site of inflammation and infection, and enhance the microbicidal ability of macrophages (Zhu and Paul, 2008).

T-helper cells depend on the antigen presenting cells (APCs) that collect antigens from their environment and display it to the CD4+ cells. These cells include dendritic cells, macrophages and B cells. After their activation by APCs they differentiate into effecter cells that are specialized in terms of the cytokines and effecter molecules that they express on their membranes or secrete and their distinct effector functions. Depending on the magnitude and pattern of T-cell receptor (TCR), co-stimulatory and cytokine signals received, T-helper cells differentiate into T-helper cell-1 and T-helper cell-2.

The Th1 subtype is characterized by predominant production of pro-inflammatory cytokines like interferon γ (IFN-γ), tumour necrosis factor-beta (TNF-β), and interleukin 2 (IL-2) (Romagnani, 1991, 1999). In the presence of IL-12 and interferon γ (IFN-γ), naïve T-cells can differentiate into Th1 cells (Kidd, 2003; Neurath et al., 2003) and therefore, the Th1 immune response is thought to be mainly chosen through circulation of IFN-gamma and IL-12 (Misra et al., 1995). The cytokines produced by Th1 cells stimulate the phagocytosis and destruction of microbial pathogens by macrophages and other lymphocytes. Interleukin-2 and IFN-gamma secreted by Th1 cells support the delayed-type hypersensitivity of pathogen (Misra et al., 1995). On the other hand, IFN-gamma inhibits the proliferation of Th2 cells, and increased concentration of IL-4 and IL-6 prevent the generation of Th1 from naïve T-cell (Kidd, 2003). Although Th1 cells are not cytotoxic, they do play a very significant role in cell-mediated immune response against intracellular pathogens and delayed type of hypersensitivity reaction (Romagnani, 1991). A Th1-dominant immune response to *M. leprae* indicates the development of a milder form of leprosy (TT) (Misra et al., 1995).

Th2 cells are involved in the humoral immune response and host defense against extracellular parasites. Th2 cells are critical for humoral immunity, especially IgE synthesis, and for favoring the differentiation of eosinophilic granulocytes (Walker and Lockwood, 2006). Th2 subtype cells are characterized by their secretion of IL-4, IL-5, IL-6, IL-10, and IL-13 (Romagnani, 1991, 1999). Th2-dominant immune response to *M. leprae* indicates development of lepromatous leprosy. Lepromatous lesions are characterized by the expression of more IL-10 and IL-4, and less IFN-γ (Pinheiro et al., 2011) with the suppression of IL-12 by IL-10.

2.2.2.3. Macrophage response

Macrophages are cells produced by the differentiation of monocytes. These cells have three major functions in inflammation – antigen presentation, phagocytosis, and immune-modulation through production of various cytokines and growth factors (Fujiwara and Kobayashi, 2005). Macrophages provide innate and acquired defense mechanism against *M. leprae* (Classen et al., 2009) and they are the primary host cells for this bacilli. For instance, in the absence of effective adaptive immune response, the *M. leprae* can multiply up to over 100 bacilli per macrophage (Scollard et al., 2006). In the defense against *M. leprae*, macrophages play an essential part in the mechanism of bacterial lyses requiring the presence of cytokines such as interleukin 2 and gamma interferon (Cuevas-Santos et al., 1998).

Macrophages are mainly activated by cytokines [IFN-γ and TNF-α] from Th1 cells and provide well established features of cellular immunity to infection with intracellular pathogens (Gordon, 2003; Magombedze et al., 2013). The activated macrophages are deactivated by anti-inflammatory cytokines (interleukin 10 and transforming growth factor ß) and cytokine antagonists that are mainly produced by macrophages (Fujiwara and Kobayashi, 2005). When the infected macrophages are inactive, *M. leprae* is able to evade the cellular immune response and replicates inside of the cell until it bursts (Magombedze et al., 2013). Without any signal from outside sources, macrophages are unable to mount any significant response to the bacterium, and the infection spreads largely unchecked (Magombedze et al., 2013). When activated through Th1 cells [and the cytokines secreted by them], macrophages then are more likely to apoptosis (thereby killing the residing bacterial load) and also activate their lysosomes to fuse with any phagosomes that might be harboring bacteria (Magombedze et al., 2013). When macrophages or dendritic cells are activated by *M. leprae* infection, several receptors and inflammatory molecules are up-regulated. Furthermore, interleukins such as IL-1, IL-2 IL-12, and IL-18 are also produced (Horwitz et al., 1984). Moreover, the activated macrophage produces TNF-α particularly in response to lipo-polysaccharides of gram negative bacteria and generally associated with resistance to *M. leprae* (Cardoso et al., 2011a). For example, serum levels of TNF-α are elevated in patients with resistant (tuberculoid) disease and with type 1 reactions, and expression of this cytokine is also increased locally in skin lesions in these manifestations of leprosy (Scollard et al., 2006). Additionally, the secreted TNF-α also involve in the activation of NK cells (Horwitz et al.,

1984). *M. leprae* induces apoptosis in macrophages in a dose-dependent manner (Maeda et al., 2005) and, in fact, this is correlated with high levels of TNF-α (Hernandez et al., 2003). It is thought that this apoptosis helps to inhibit bacterial proliferation of the mycobacterium since mycobacteria are not efficient at replicating outside of the cell (Hernandez et al., 2003). Researchers also claim that this may be one of the main differences between TT and LL leprosy (Hernandez et al., 2003). Macrophages can inhibit or kill pathogens through the generation of reactive oxygen intermediates and of reactive nitrogen intermediates.

2.2.2.4. Natural killer cell response

Activation of NK cells is induced by intracellular bacteria by inducing the expression of NK cell–activating ligands on infected cells. Furthermore, NK cell–activating cytokines like - IL-12 and IL-15 that produced by dendritic cells and macrophages can also induce the activation process (Adib-Conquy et al., 2014). The activated NK cells produce large amounts of IFN-γ and other cytokines which provide adaptive immune response. However, NK cells provide an early stage of defense against the microbes, before the development of adaptive immune system (de la Barrera et al., 2004). Furthermore, the produced IFN-γ in turn activates macrophages and promotes the killing of the phagocytosed bacteria. In both innate and adaptive immune response cases, NK cells play a key role at fighting against *M. leprae* infection. NK cells involve in the activation of T-cells and promote the differentiation of Th1 cells these NK cells activities are the vital part of the defense system against *M. leprae* infection (de la Barrera et al., 2004). NK cells also secrete IL-13, though in smaller amounts compared to T-cells (de la Barrera et al., 2004). This is interesting, because IL-13 is a known cytokine inhibitor, and generally stops any further IFN-gamma production - and therefore essentially stops the inflammatory process (de la Barrera et al., 2004).

2.2.3. Genetic susceptibility to leprosy

Host genetic factors are largely involved in the susceptibility to leprosy. Various genes and genomic regions in the human genome have been suggested to have a role in the susceptibility to leprosy particularly, those do influence the human immune response to *M. leprae* (Scollard et al., 2006). These have been supported by data from different sources like: complex segregation analysis, twin studies, candidate gene association studies, single nucleotide polymorphism (SNP) association studies, and genome-wide association studies

(GWAS). Candidate gene strategies using both case-control and family-based designs, as well as large-scale approaches such as linkage and gene-expression genomic scans and, more recently, genome-wide association studies, have refined and enriched the list of genes highlighting the most important innate and adaptive immune pathways associated with leprosy susceptibility or resistance (Cardoso et al., 2011b).

Many studies have demonstrated the presence of high concordance rate for leprosy among monozygotic compared with dizygotic twins (Fava and Mira, 2012). For instance, a study by Chackravartti and Vogel showed a 3-fold greater concordance rate for the type of leprosy disease in monozygotic twins than dizagotic twins (Chakravartti and Vogel, 1973). Similarly, a number of linkage and candidate gene studies in many populations of the world have pointed out associations of the major histocompatibility complex (MHC) class I and II loci with leprosy susceptibility (Fitness et al., 2002). These studies especially indicated the association between HLA-DR2 alleles and the disease (Fitness et al., 2002). Single nucleotide polymorphism association study by Alcais and colleagues also indicated the co-relation between early onset of leprosy and low lymphotoxin-α (LTA)-producing allele (Alcais et al., 2007). Recent study of genome wide linkage analysis has identified a new linkage locus on chromosome 2p14 for leprosy Pedigrees from China (Yang et al., 2012). On the other hand, fine mapping of the chromosome 6 locus identified polymorphic risk factors in the shared promoter region of two genes, PARK2 and PACRG (Mira et al., 2004). The association within this region was replicated in a large set of Brazilian leprosy cases and controls (Frodsham and Hill, 2004). These two genes are the most important candidate genes for leprosy susceptibility. Both are expressed in the host cells [Schwann cells and macrophages] of *M. leprae*. PARK2 gene codes for an E3-uniquitin ligase and associated with Parkinson disease whereas PACRG forms part of a molecular chaperone complex (Mira et al., 2004). Additionally, variant form for the different alleles of the vitamin D receptor (VDR) gene were also associated with the susceptibility to tuberculoid or lepromatous disease (Fitness et al., 2002).

Table 4: Some of the candidate genes and genomic regions that associated with leprosy

Genomic region	Gene	Description	Reference
10p13	MRC1	Encode for mannose receptor protein [phagocytic receptor], found to be associated with multibacillary leprosy and leprosy overall.	(Misch et al., 2010)
6q25	PARK2 PACRG	PARK2 is expressed by both Schwann cells and macrophages. It is an ubiquination E3 ligase and is involved in the delivery of polyubiquinated proteins to the proteosome complex involved in protein degradation	(Mira et al., 2004)
4q32	TLR2	These toll-like receptor1 or 2 are strongly expressed on monocytes and dendrite cells (DC) in TT compared to in the LL and in in-vivo the M. liprae lipoproteins could activate monocytes and monocyte derived DCs through TLR2	(Krutzik et al., 2003)
4p14	TLR1		
6p21.3	LTA	LTA gene encodes for lymphotoxin α which is a chemokine secreted by lymphocytes and natural killer cells. Studies indicated the linkage of this gene to susceptibility of leprosy.	(Fitness et al., 2002)

2.2.4. Mechanisms of nerve injury

Infection and damage of peripheral nerves is the most serious and unique property of *M. leprae* (Scollard et al., 2006). Neuropathy (nerve damage) in leprosy arises not only from the infection of peripheral nerves by *M. leprae* but usually develops from the inflammatory and immunologic responses to this pathogen (Harboe et al., 2005).

2.2.5. Epidemiology of leprosy

Leprosy is one of the worldwide health problems, particularly, in Africa, Asia, and Latin America. Globally, 232,857 new cases (with 4 new case detection rate per 100,000 population) were diagnosed and reported to World Health Organization (WHO) in 2012 from 115 countries (WHO, 2013). The distribution of new cases detected in this year shows – 9 percent from 25 African countries, 16 percent from 28 American countries, 2 percent each from Eastern Mediterranean and Western Pacific, and the largest percentage (71%) was accounted for 11 South East Asian countries. According to this WHO annual report, leprosy prevalence and new case detection increased in 2012 as compared to the previous reports. However, the general figure of leprosy epidemiology indicates the declining of the prevalence as a consequence of multidrug therapy [MDT] (WHO, 2013). Among 232,857 new cases detected in 2012, sixteen countries reported ≥1000 new cases and they accounted for 95% of the cases (WHO, 2013), Table 5.

Table 5: Number of newly detected cases over the last five years (WHO, 2013)

Country	2008	2009	2010	2011	2012
Bangladesh	5 249	5 239	3 848	3 970	3 688
Brazil	38 914	37 610	34 894	33 955	33 303
China	1 614	1 597	1 324	1 144	1 206
Cote d'Ivoire	998	884	NR	770	1 030
DR Congo	6 114	5 062	5 049	3 949	3 607
Ethiopia	4 170	4 417	4 430	NR	3 776
India	134 184	133 717	126 800	127 295	134 752
Indonesia	17 441	17 260	17 012	20 023	18 994
Madagascar	1 763	1 572	1 520	1 577	1 474
Myanmar	3 365	3 147	2 936	3 082	3 013
Nepal	4 708	4 394	3 118	3 184	3 492
Nigeria	4 899	4 219	3 913	3 623	3 805
Philippines	2 373	1 795	2 041	1 818	2 150
Sri Lanka	1 979	1 875	2 027	2 178	2 191
South Sudan	-	-	-	1 799	1 801
Tanzania	3 276	2 654	2 349	2 288	2 528
Total	**231 047**	**225 442**	**211 261**	**210 655**	**220 810**
Global Total	**249 007**	**244 796**	**228 474**	**226 626**	**232 857**

Very few new leprosy patients are registered in developed countries. When leprosy is detected in these countries, it is primarily found among immigrants from countries where the disease is still endemic (Saikawa, 1981). There is an association between the incidence of leprosy and socioeconomic factors such as gross national product (GNP), personal housing expenditures and the number of persons per household, suggesting that improvements in socioeconomic conditions greatly contribute to the reduction of leprosy (Saikawa, 1981).

In Africa, the registered prevalence of leprosy declined from 3.53 cases per 10 000 population in 2010 to 3.05 cases per 100 000 population in 2012(WHO, 2013). Although the number of new cases decreased within the past 7-years [from 46 918 in 2004 to 20 213 in 2011] it showed increment to 20 599 in 2012 (WHO, 2013). The proportion of multi-bacillary leprosy among the newly detected cases ranged from 66.3% in Democratic Republic of the Congo and 92.3% in Burkina Faso. The proportion of children among new cases ranged from 1.3% in Niger to 24.5% Cameroon. Similarly, the proportion of new case leprosy patient females ranged from 20.8% in Madagascar to 49.6% in Sierra Leone (WHO, 2013).

The registered new cases of leprosy have been increasing in Ethiopia. In 2006 was 4,092, it is increased to 5,303 in the first quarter of 2011(WHO, 2013). However, this number declined to 3,776 in 2012. According to the WHO report of leprosy prevalent in 2012, the number of new cases of MB leprosy is 3 420; females are 912; children are 260; and grade 2 disabilities are 261. Researches indicated that in developing countries like Ethiopia, socioeconomic factors, mitigation of overcrowding and poverty, as well as poor nutritional and hygiene habits have great contribution for the increment of leprosy prevalence (Saikawa, 1981).

2.2.6. ABO blood group and leprosy

ABO blood group is the most important blood type in the clinical practice of blood transfusion and transplantation. Most people exhibit the standard blood group A, B, AB and O phenotypes. Besides, the common A1 and A2 subgroups, a number of other rare subgroups exist that are characterized phenotypically by the quantity and distribution of A and B antigens on red blood cells [RBC]. The regulation of ABO blood group system is under the control of ABO gene expression (Kominato et al., 2005). Research on this blood group has been of enormous interest, due to its medical importance and the linkage of person's ABO

phenotypes to the susceptibility of many diseases. The association between ABO blood groups and leprosy has been conducted in different countries at different time by different researchers. Most of the studies were done in Argentina, Japan, Latvia, Egypt, Brazil, China, and India (Salzano, 1967); and the data of 27 series were pooled together and analyzed in 1967 by Salzano and the result showed low frequency of O and relatively high frequency of B in lepromatous leprosy patients. Another combined data of 31 series analysis also indicated slightly significant association of A blood type to leprosy compared to O blood type (Vogel, 1968). This analysis also indicated lower frequency of B in leprosy patients compared to O blood type but this was not significant (Vogel, 1968). Similarly, there is slight and significantly higher frequency of A blood type in lepromatous leprosy patients than O blood type (Vogel, 1968). However, a study in Bangladesh among 100 leprosy patients indicated the absence of apparent difference in ABO blood group pattern between leprosy patients and general population (Ahamad et al., 2008).

2.2.7. Hp polymorphism and leprosy

Due to its multifunction, haptoglobin gene has received the attention of many researchers. Particularly, the important interaction between haptoglobin, immunology, and susceptibility to leprosy made such kinds of studies to get the interest of many researchers. A lot of investigations indicated the association between haptoglobin gene polymorphisms and several infectious and non-infectious diseases. In the same way, many studies have been done in different countries to correlate the different form of haptoglobin genotype with the spectrum of leprosy (Gupta et al., 2007; Saoji et al., 1980; Sritharan et al., 1981; Walter et al., 1970; Walter et al., 1972). Some of these studies have got significant correlation between leprosy and haptoglobin genotype particularly anhaptoglobinemia (Hp^oHp^o, which indicates the absence of haptoglobin in the serum due to the deletion of Hp gene) (Gupta et al., 2007; Saoji et al., 1980).

3. Objectives of the study

3.1. General objective

- ✓ The general objective of this study is to assess haptoglobin and ABO blood group variation among leprosy patients from ALERT Hospital as compared to healthy controls.

3.2. Specific objectives

- ✓ To identify Hp genotype frequency distribution among leprosy patients
- ✓ To identify plasma Hp concentration difference along leprosy spectrum
- ✓ To study ABO blood group distribution among leprosy patients

4. Materials and methods

4.1. Study population

The study participants were leprosy patients who were referred to ALERT hospital from different regions of Ethiopia and clinically diagnosed to be leprosy patient. A total 109 new case leprosy patients who came to ALERT hospital from the beginning of August 2013 to the late October 2013 were involved in the study. The desired sample size was obtained by purposive sampling technique. The identified cases who met the proposed inclusion and exclusion criteria were requested to sign consent form before participating in the study.

Additionally, the study included healthy controls those who did not have related health problem and met both the inclusion and exclusion criteria of the study. They were recruited from Ayer-Tena preparatory school depending on their socio-demographic backgrounds. After obtaining permission from the School Administrator, 300 relevant individuals were selected from master lists based on their sex, age, ethnic group, and place of birth. The selected individuals were called for a short time meeting and the objectives of the study were described and discussed. From 243 individuals who attended the meeting 194 were volunteers to participate in the study. Then, 108 individuals were selected by lottery sampling method.

4.2. Study design

A case-control study design was applied and the ratio of controls to cases was approximately equal (Etzioni et al., 1999).

4.3. Study site

The study subjects were recruited from All Africa Leprosy Tuberculosis Rehabilitation Research Training Center (ALERT) hospital, Addis Ababa, Ethiopia. ALERT is situated in southwest of Addis Ababa on the way to Jima. The Hospital is one of the largest hospitals in the country which mainly provides training for both genders in many aspects of leprosy disease including prevention, treatment, and rehabilitation in an Africa context of environment. Since 2002, the Hospital has been serving as a referral and teaching for leprosy, skin, TB, HIV, and Ophthalmology diseases under the administration of the Ministry of

Health of the Federal Democratic Government of Ethiopia. Currently, more than 500 leprosy patients visit ALERT Hospital every year from different regions of Ethiopia.

The study controls were recruited from Ayer-Tena Preparatory School which is located in southwest of Addis Ababa on the way to Jima and at about 2km from ALERT Hospital. The school community consists of 4300 members who are teachers, administrative workers, regular and night division students.

4.4. Sample size and sample size determination

It is common to sample equal numbers of cases and controls (Etzioni et al., 1999), or, four to five controls per case when controls are easier to obtain and when the cost of including additional controls as well as duration of study are negligible (Etzioni et al., 1999). In this study, the ratio of controls to cases was designed to be approximately equal. The sample size was computed using sample size calculation software for genetics studies [QUANTO, Version 1.2]. QUANTO is a program for computing either power or required sample size for association studies of genes, environmental factors, gene-environment (GxE) interaction, or gene-gene (GxG) interaction. The program is written in C++ and is designed to run under window 95, 98, 2000, XP, and NT (Gauderman and Morrison, 2007). Thus, the required sample size for this study was calculated by considering the following assumptions and facts.

- Inheritance Model – Additive
- Leprosy prevalence – 0.57
- Allele frequency – 0.54
- Level of significance (α) – 5%, 2-sided and
- Study design – case-control

  Based on the above assumptions 92 case-control pairs were determined and 17% (16 pairs) of none response rate was added to get 108 total samples.

INCLUSION CRITERIA:

Individuals were eligible for inclusion in the study if they were:

- Leprosy patients (except controls)
- volunteer to participate in the study
- 18 year of age or above
- Any ethnic group of Ethiopia

EXCLUSION CRITERIA:

Individuals were excluded from this study if they were:

- without relevant health problem except volunteer controls
- pregnant and/or under weight
- hemophilic patient
- with serious chronic illness
- children (< 18 years)
- differ from the socio-demographic characteristics of cases (for controls)
- not consent to participate in the study

4.5. Sample quality management

In the study, 2mL to 3ml venous blood samples were collected from 109 volunteer leprosy patients and 108 healthy controls by qualified health professional with strict adherence to safety and privacy regulations. The whole blood samples were collected in tubes containing anticoagulant (EDTA). The blood samples were centrifuged at 3000rpm for 10 minutes to prepare plasma and Buffy coat. Then, the plasma was carefully aspirated without disrupting the white blood cells layer and added into the correspondingly labeled 2ml cryo-tubes. Similarly, the Buffy coat layer (with some overlying plasma and underlying red blood cells) was transferred into 15ml blue cap tubes (which was adequately labeled with relevant information) using 1ml Pasteur pipette. The samples were maintained at room temperature (18-24^{o}C) throughout sample processing (centrifugation, plasma aspiration, Buffy coat separation, and ABO blood group examination). Then both the Buffy coat and plasma were stored at -80^{o}C till DNA extraction and ELISA assay, respectively.

4.6. Leprosy diagnosis

The whole diagnostic processes were done by dermatologists, and technicians experienced in leprosy diagnosis. Leprosy patient identification and clinical examination were conducted at ALERT hospital laboratory. The diagnosis of leprosy was based on different clinical parameters which involved in:

- History assessment about the presence and duration of lesions, nerve pain, numbness and tingling, weakness, ulcer and injuries, eye pain and worsening vision.
- Careful entire skin examination for anaesthetic lesions which includes macules, papules, plaques, nodules, urticaria – like lesions and smooth infiltrations. Skin examination also included – looking for loss of sensation, hair, pigmentation, and sweating.
- Nerve palpation which involved in the assessment of the most commonly affected nerves such as: ulnar, median, radial cutaneous, common peroneal (lateral popliteal) and posterior tibial nerves, the sural nerve, the 5^{th} and 7^{th} cranial nerves, and the greater auricular nerve.
- Nerve function assessments like nerve function impairment [NFI] testing of motor and sensory function by voluntary muscle testing – sensory testing [VMT-ST]
- In disability assessments, the disability of the individual organ was assessed and recorded by grading at the time of first examination. The disability of hands and feet was tested for their sensory loss and voluntary muscle power. While the disability of eyes was examined for vision, lid gap, and blinking. Then the individual disability grades for the two eyes, two hands and two feet were graded based on WHO disability grading system where each hand, foot, and eye is graded as 0, 1, or 2 and these grades are summed bilaterally for a maximum score of 12.

In this study, the clinical diagnosis of leprosy was also supported by bacteriological examination of acid-fast bacilli (AFB) in slit skin smears. The skin smear samples of patients were taken from suspected skin lesions particularly from the most active looking edge of the lesion and stained by Ziehl-Neelsen staining method for bacteriological reading and gradation of smears (Jopling and Mcdougall, 1999). After staining, the slides were examined using a 100x oil immersion lens. Following this, the percentage of solid staining bacilli (the viable leprosy bacilli) which appeared uniformly stained was recorded as morphological index (MI). Moreover, the total number of AFB in the skin was quantified and recorded as

bacterial index (BI). The BI was obtained by adding the scores from each site and dividing by the number of sampled sites. It was then scored in the range from 0+ to 6+ depending on the number of bacilli seen in an average microscopic field using an oil immersion lens or high power field.

The obtained clinical features (physical examination) and laboratory investigations (slit skin smear test) were used to group patients into tuberculoid (TT), Borderline Tuberculoid (BT), Borderline (BB), Borderline Lepromatous (BL), and Lepromatous (LL) according to Ridley and Jopling scaling (Table 6). Patients who had a clinical form of disease limited to nerve involvement alone were grouped into pure neural leprosy.

Table 6: clinical aspects of the Ridley and Jopling classification of leprosy according to their immunity (Mathur et al., 2011)

Observation or Test	Type of leprosy				
	TT	BT	BB	BL	LL
Number of Lesions	Single Usually	Single or Few	Many	Many	Many
Size of Lesion	Variable	Variable	Variable	Variably	Small
Surface of Lesion	Very dry, sometimes scaly	Dry	Slightly Shiny	Shiny	Shiny
Sensations in Lesion (Not face)	Absent	Moderately or markedly diminished	Slightly or moderately diminished	Slightly diminished	Not affected or minimally affected
Hair growth In lesions	Absent	Markedly diminished	Moderately diminished	Slightly diminished	Not affected
AFB in Lesions	Nil	Nil or scanty	Moderate numbers	Many	Very many
AFB in nasal Scraping or in nose blows	Nil	Nil	Nil	Usually nil	Very many
Nerve involvement	many have one enlarged peripheral nerve and occasionally present as a mononeuralpathy	Asymmetrical multiple nerve involvement	Asymmetrical multiple nerve involvement	Wide spread, nerve thickening, sensory and motor loss	Wide spread, enlarged nerve, anesthesia occurs in late in disease
BI	0 to 1	0 to 2	2 to 3	1 to 4	4 to 6

4.7. Enzyme Linked Immuno-Sorbent Assay

Total plasma haptoglobin level was quantified by enzyme-linked immunosorbent–assay (ELISA) method [ab108856 haptoglobulin human ELISA kit, abcam] based on the instruction provided by the manufacturer. The frozen samples were thawed and diluted 1:2000 with diluents. All the reagents were brought to room temperature and freshly diluted as follow: 1) the diluent (10X) was diluted 1:10 with reagent grade water; 2) haptoglobulin standard solution of 40µg/ml was prepared by re-suspending 20µg of lyophilized human haptoglobin standard in 0.5ml of diluent. After the standard was allowed to completely dissolve for 10 minutes at room temperature, standard points were prepared by serial dilution of the standard solution as shown in table 6; 3) two fold stock solution of biotinylated Haptoglobin was prepared by diluting biotinylated Haptoglobin (2X) by diluting with 4ml diluent and then allowed to mix thoroughly for 10 minutes with gentle agitation prior to further 1:2 dilution with diluents; 4) the 20X wash buffer was diluted with reagent grade water in 1:20 proportion; 5) a 100 fold concentrated Streptavidin-Peroxidase Conjugate (SP Conjugate) was diluted into 1:100 with diluent.

Table 7: Serial dilution of the human Haptoglobin standard solution

Standard points	Dilution	[Haptoglobin] (µg/ml)
P1	1 Part Standard (20µg/ml)	20.000
P2	1 part P1 + 3 part Diluent	5.000
P3	1 part P2 +3 part Diluent	1.250
P4	1 part P3 +3 part Diluent	0.313
P5	1 part P4 +3 part Diluent	0.078
P6	Diluent	0.000

After all the reagents were prepared and set at room temperature, 25µl of each sample was added separately to each 92 wells, however, the same volume (25µ) of standard was added to each of the last six wells starting from the most concentrated standard to the least. Then, 25µl of biotinylated Haptoglobin was added to each well and mixed gently. After biotinylated Haptoglobin addition, the wells were covered with a sealing tape and incubated at room temperature for one hour. Following incubation, manually, the wells were washed five times

with 200µl wash buffer by inverting the plate each time to decant the contents and by tapping it 5 times on the absorbent paper towel to completely remove the liquid. Then, 50µl of Streptavidin-Peroxidase Conjugate was added to each well and incubated for 30 minutes at room temperature and then washed five times as mentioned above. Following washing, 50µl of Chromogen Substrate was added to each well and incubated for about 10 minutes till the optimal blue color density was developed. Then, 50µl stop solution was added to each well (the color changed from blue to yellow) and soon the absorbance was read on micro-plate reader at a wavelength of 450nm. Finally, the concentration of Hp was determined using a standard curve of purified Hp.

4.8. ABO blood group serology

4.8.1. ABO blood grouping

The ABO blood grouping was done by antigen-antibody agglutination test using slide method and commercially available anti-A and anti- B monoclonal antibodies (Tulip Diagnostics, India). Following the preparation of suspension of test red blood cells from EDTA tube, 1 volume of anti-A or anti-B reagent and 1 volume of test red cell suspension were placed on a labeled glass slide. Then the reagent and the cells were mixed over an area of about 20x40mm using a clean applicator stick. The slide was slowly tilted back and forth for about 30 seconds with occasional further mixing during the 2 minutes of period by maintaining the slide at room temperature (18-24^{o}C). And then, agglutination was read macroscopically after 2 minutes over a diffused light with careful investigation to avoid mistakes due to fibrin strands and weak reactions.

4.8.2. ABO blood group phenotypes, genotypes, and allele frequencies

The ABO blood group phenotype of cases and controls were obtained from serologic examination. Then their phenotypic proportions were calculated using these serological data. However, the population phenotypic frequencies were calculated from secondary data of national blood bank of Ethiopia which had been used by Haftom to analyze risk factors among blood donors (Haftom, 2011).

The estimate of allele frequencies of all data was determined by maximum-likelihood estimation of allele frequency using the formula suggested by Hedrick as shown below(Hedrick, 2005).

$$p = 1 - \sqrt{\frac{n_B + n_O}{N}}$$

$$q = 1 - \sqrt{\frac{n_A + n_O}{N}}$$

$$r = \sqrt{\frac{n_O}{N}}$$

Where:

- $N = Total\ individuals$,
- $n_A = individuals\ with$ A phenotype,
- $n_B = individuals\ with$ B phenotype, and
- $n_O = individuals\ with$ O phenotype

Based on the results from Hedrick (2005) allele frequency (above) the genotypic frequency and the number of individuals having each genotype (AA, AO, AB, BB, BO, and OO) was calculated using the following formula (Dempster et al., 1977).

$N_{AA} = n_A\left(\frac{p^2}{p^2+2pr}\right)$; $N_{AB} = n_{AB}$

$N_{AO} = n_A\left(\frac{2pr}{p^2+2pr}\right)$; $N_{OO} = n_{OO}$

$N_{BB} = n_B\left(\frac{q^2}{q^2+2qr}\right)$; $N_{BO} = n_B\left(\frac{2qr}{q^2+2qr}\right)$

$n_A, n_B, n_{AB}, n_{OO} = number\ of\ individuals\ with\ phnotype$
$A, B, Ab, and\ O\ respectively$

$N_{AA} = individuals\ with\ AA\ genotype;\ N_{BB} = individuals\ with\ BB\ genotype$

$N_{AO} = individuals\ with\ AO\ genotype;\ N_{BO} = individuals\ with\ AO\ genotype$

$N_{BO} = individuals\ with\ AO\ genotype$

$p = frequency\ of\ I^A : q = frequency\ of\ I^B : r = frequency\ of\ I^B$

4.9. Haptoglobin genotyping

4.9.1. DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes by standard non-organic (proteinase K and salt out) extraction procedure (Miller et al., 1988). The frozen blood (rich in WBCs) was thawed at room temperature. Then the red blood cells were lysed in 15ml polypropylene tube with 3ml of EL-buffer (0.155M NH_4Cl, 10mM KHCO3, 10m MEDTA and PH 7.3). The mixture of EL-buffer and Buffy coat was maintained in ice for at least 15 minutes by mixing methodically by inverting. Then it was centrifuged at 4000 rpm for 10 minutes and the supernatant was poured off into an appropriate biohazard container by maintaining the pellet. The pellet was re-suspended by 3ml KL-buffer (10mM Tris, 2mM EDTA, 0.4 M NaCI, pH 8.2) and shaken vigorously to collapse the pellet. Following, the cell lysates were digested overnight at 37^{o}C with 200µl of proteinase K (20mg/ml) and 200µl of 20% SDS.

On the second day, the mixture was checked for complete digestion of the pellet and 1ml of 5M NaCl was added and shaken vigorously and then centrifuged at 3000 rpm for 10 minutes. The supernatant containing the DNA was transferred into another 15ml polypropylene tube and exactly two volume of room temperature 96% ethanol was added and then the tube inverted several times until the DNA precipitated. The precipitated DNA was taken by Pasteur pipette and transferred into 1.5ml eppendorf tube. Then it was washed by 400µl of 70% ethanol and centrifuged at 6000rpm for 10 minutes. The supernatant was poured off and the pellet was allowed to air-dry at room temperature. Finally it was dissolved in 50 to 100µl TE-buffer (10mM Tris-HCL, 0.1mM EDTA, pH 7.5) and stored at +4^{o}C

4.9.2. Haptoglobin genotype determination

The common alleles of Haptoglobin gene were amplified by using allele specific polymerase chain reaction technique. The reaction was carried out using four oligo-nucleotide primers adapted from Koch et al. (2005). The primers were synthesized by Applied Bio-systems (Kenya). The lyophilized (130nmol) primers were re-suspended by 1.3ml of nuclease free water (Qiagen).

Table 8: The four primers used to amplify the common alleles of haptoglobin gene

Primer Name	Primer Nucleotide Sequence
A	5'-GAGGGGAGCTTGCCTTTCCATTG-3'
B	5'-GAGATTTTTGAGCCCTGGCTGGT-3'
C	5'-CCTGCCTCGTATTAACTGCACCAT-3'
D	5'-CCGAGTGCTCCACATAGCCATGT-3'

Two PCR reaction mixtures per sample with different primer combinations were used to amplify the target nucleotide sequences. In the first reaction mixture, primer A and B were used to amplify either 1757bp of Hp1 allele specific sequence or 3481bp of Hp2 sequences. The second reaction mixture was allowed to contain primer C and D to amplify a 349bp nucleotide sequence which is a section of Hp2 and specific to this allele.

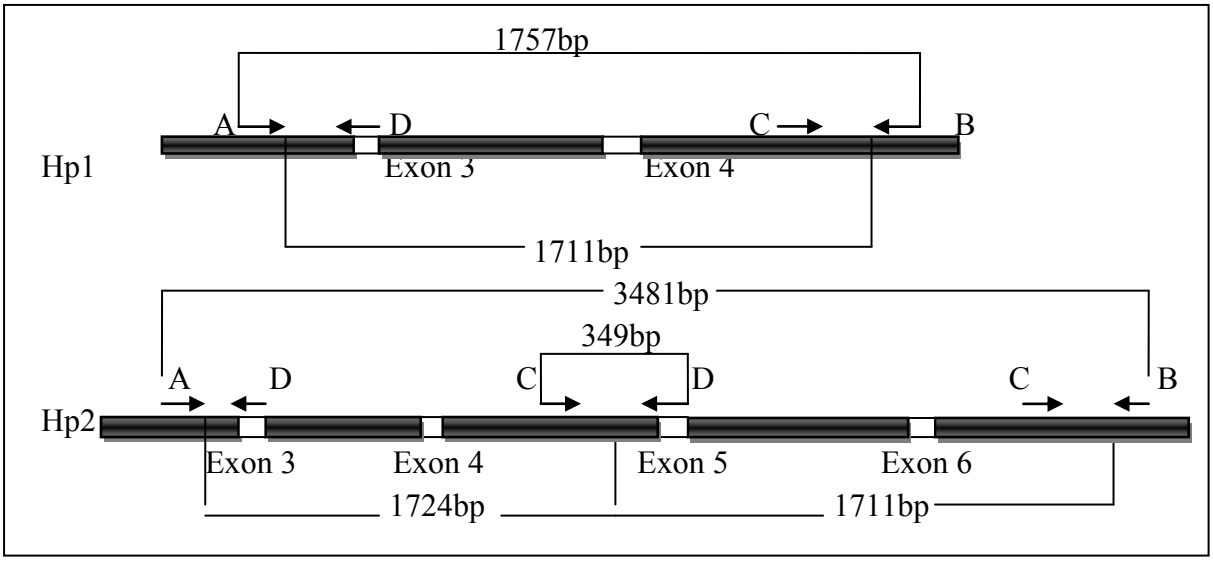


Figure 3: Partial structure of Haptoglobin alleles, Hp 1 and Hp 2 and annealing sites of primer A, B, C and D (re-drown from (Koch et al., 2002).

Polymerase chain reaction was conducted in thermal cycler TC-512. A total of 20µl PCR reaction mixture was used per reaction. Each reaction mixture was consisted of HotStarTaq Master Mix Kit (providing a final concentration of 1.5mM $MgCl_2$ in the final reaction mix, 2.5 units HotStarTaq DNAP, 1xPCR Buffer, and 200µM of each dNTP) [synthesized by Qiagen], primers (0.5pmol/µl), template DNA (≈1µg/50µl), and nuclease free water (Qiagen), Table 9.

Table 9: PCR reaction components to amplify the two common alleles of Haptoglobin gene

Components			Volume/reaction	Final concentration
Hotstar taq master mix			10µl	2.5 units HotStarTaq DNAP
				1xPCR Buffer
				200µM of each dNTP
Primer A	Or	Primer C	1µl	0.5µM
Primer B		Primer D	1µl	0.5µM
RNase-free water			7µl	-
Template DNA			1µl	≈ 1µg/25µl reaction
Total			20µl	-

Each reaction mixture was set to be amplified at different PCR amplification programs based on the primer combination in the mixture. The first PCR reaction mixture containing A and B was set for touchdown PCR program under the conditions listed in Table 10. The second PCR reaction mixture containing primer C and D used Two Step PCR program as shown in Table11.

Table 10: PCR reaction conditions for the reaction mixture containing primer A and B

95°C	15minutes	1 cycle
95°C	30seconds	15 cycles
69°C to 54°C	45seconds	
72°C	1minute	
95°C	30 seconds	35 cycles
55°C	45 seconds	
72°C	1 minute & 30se	
72°C	7minutes	1 cycle
4°C	Forever	(pick up samples)

Table 11: PCR reaction conditions for the reaction mixture containing primer C and D

95°C	15 minutes	1 cycle
95°C	45 seconds	35 cycles
69°C	2 minutes	
72°C	7 minutes	1 cycle
4°C	Forever	(pick up samples)

4.9.3. Gel electrophoresis

The amplification products from the two reactions (Reaction-1 with primer A & B; Reaction-2 with primer C & D) were combined and differentiated by electrophoresis using 1% agarose gel, 1xTAE buffer, Ethidum Bromide, loading dye, and 100V for 45 minutes. One gram of Agarose powder was measured and dissolved in 100ml of 1X TAE buffer (40mM of tris-base pH7.6, 10mM of EDTA pH8, and 20mM of glacial acetic acid) by heating in microwave till it completely dissolves. Following the cooling of agarose solution to about 50-55^{o}C, 6µl of Ethidum Bromide (10mg/ml) was added to the agarose solution and poured in gel casting tray which was arranged with combs and then allowed to cool on casting tray until it was solid. Then, the solidified gel was placed in the electrophoresis Pan which contained enough amount of TAE buffer (1X) and the comb was pulled out carefully. Following this, 1X sample loading dye (6X: 6ml glycerol, 1.2ml EDTA (0.5M), and 2.8ml double distilled water) was added to each 20µl of PCR reaction and carefully 6µl of the mixture was dispensed into the separate wells in the gel. In the first Lane, 5µl of DNA size marker (1kb, Sigma-Aldrich) was dispensed before loading the samples. Finally, the electrode wire was connected to the power supply at 120V (constant voltage) and run for a minimum of 45 minutes.

4.10. Statistical analysis

Allele frequencies were calculated under the assumption of Hardy–Weinberg equilibrium and expressed as percentages. Chi-square test and likelihood ratio were used to compare observed allelic and genotypic frequency distributions of the blood group and Haptoglobin. Shapiro-Wilk test and an inspection of the Skewness and Kurtosis measures and standard errors as well as visual inspections of their histogram, normal Q-Q plots, and pox plots were used to test for the normal distribution of continuous data.

In order to check for all possible differences among the groups, analysis of variance (ANOVA) was used. Comparisons between groups were made using Student's t- test for normally distributed continuous variables and Mann-Whitney test for non-parametric test. A p values less than 0.05 were considered statistically significant and all the analysis were done

with SPSS version 21 and STATA version 12.1 software. The OD data were to concentration using Graphpad prism 6 Demo Software.

4.11. Ethical consideration

The protocol was approved by the AHRI/ALERT ethical review committee of the Armauer Hansen Research Institute and Ethical Review Committee of College of Natural Science, Addis Ababa University. Moreover, full consent forms were signed by the subjects after the nature and motif of the study was clearly explained to them.

5. Results

5.1. Demographic characteristics

A total of 217 [109 Leprosy patients and 108 non-leprosy healthy controls] individuals were recruited during the study period: out of this total, 135 (62.2%) were males and 82 (37.8%) were females with age range of 59. Participants between age of 19 and 55 years comprised 94.9% (206) of the total. The details are shown in Table 12.

Table 12: Demographic characteristics of studied populations

		Study Populations Category		Total
		Case	Control	
Gender	male	67_a	68_a	135
	female	42_a	40_a	82
Total		109	108	217
Age category	15 to 24	32_a	37_a	69
	25 to 34	31_a	41_a	72
	35 to 44	20_a	16_a	36
	45 to 54	13_a	14_a	27
	55 to 64	8_a	0_b	8
	>64	5_a	0_b	5
Total		109	108	217
Place Of Birth	R3	54_a	52_a	106
	R4	46_a	38_a	84
	R7	9_a	8_a	17
	R14	0_a	8_b	8
	Other	0_a	2_a	2
Total		109	108	217

Each subscript letter denotes a subset of Category Of Study Participants categories whose column proportions do not differ significantly from each other at $\alpha = 0.05$ level.

R3=Region-3, R4=Region-4, R7=Region-7, R14= Addis Ababa

5.2. ABO blood group

The common ABO blood group phenotypic distribution pattern between studied populations and the comparison of studied populations with general population data from Ethiopian National Red Cross is presented in Table 13. The distribution of ABO phenotypes in the total samples was 44.2% (96/217), 30% (65/217), 20.7% (45/217) and 5.1% (11/217) for groups "O", "A", "B" and "AB" respectively. Blood type "O" was the most frequent and "AB" was the least frequent phenotypes in both leprosy patients as well as in healthy controls.

Table 13: The distribution of ABO blood group phenotypes among studied populations

			Study Populations Category			Total
			Case	Control	Population	
ABO Blood Group	A	N	32	33	4415	4480
		% within Study Populations Category	0.7	0.7	98.5	100
		% within ABO Blood Group	29.4	30.6	30.9	30.9
		% of **Total**	0.2	0.2	30.4	30.9
	B	N	25	20	3522	3567
		% within Study Populations Category	0.7	0.6	98.7	100
		% within ABO Blood Group	22.9	18.5	24.7	24.6
		% of **Total**	0.2	0.1	24.3	24.6
	AB	N	5	6	381	392
		% within Study Populations Category	1.3	1.5	97.2	100
		% within ABO Blood Group	4.6	5.6	2.7	2.7
		% of **Total**	0.0	0.0	2.6	2.7
	O	N	47	49	5969	6065
		% within Study Populations Category	0.8	0.8	98.4	100
		% within ABO Blood Group	43.1	45.4	41.8	41.8
		% of **Total**	0.3	0.3	41.2	41.8
Total		N	109	108	14287	14504
		% within Study Populations Category	0.8	0.7	98.5	100
		% within ABO Blood Group	100	100	100	100
		% of **Total**	0.8	0.7	98.5	100

The proportion of "A", "B", "AB", and "O" in healthy controls was not significantly differ from the corresponding proportions of data obtained from Ethiopian National Red Cross (Pearson Chi-square test; $X^2_{(3)} = 5.30$, p = 0.15, 2-sided).

Table 14 depicts the distribution of allele frequencies of ABO in the studied populations and in the general population data from Ethiopian National Red Cross. The distribution of i^O, i^A and i^B alleles in leprosy patients was 66% (72/109), 14.7% (16/109), and 19.3% (21/109), respectively. Similarly, the proportion of i^O, i^A and i^B within the pooled data of controls and general population was 66.8% (9610/14395), 18.5% (2664/14395) and 14.7% (2121/14395), respectively. The whole data (cases, controls and population) exhibited allele frequency in the order of $i^O > i^A > i^B$. No difference in the study populations with respect to ABO allele distribution was observed in this study (2-sided Pearson chi-square test; $X^2(2) = 0.04$, P = 0.98).

Table 14: ABO blood group allele frequencies among studied group

		Study Populations Category		
		Case freq(N)	Control freq(N)	Population freq(N)
Type of allele	i^A	.190(21)	.200(22)	.185(2642)
	i^B	.150(16)	.130(14)	.148(2107)
	i^O	.660(72)	.670(72)	.668(9538)
Total		1.000(109)	1.000(108)	1.000(14287)

The genotypic frequencies of ABO blood group in 217 blood donors and 14287 individuals are summarized in Table 15. The data showed that "OO" comprised the highest genotypic frequency in all studied individuals. "OO" accounts for 43.6% (47/109) in leprosy patients, 44.9% (49/108) in health control individuals, and 44.6% (5969/14287) in the general population. "AO" was the second most frequent genotype in both leprosy patients (25.1%) and health controls (26.8%) as well as in the population (24.7%). The third most frequent genotype was "BO", which was found in 19.8% of leprosy patients, 17.4% of studied health individuals, and 19.8% of population. Low frequencies were detected for "AA", "AB", "BB" genotypes comprising less than 6% in each group.

Table 15: Genotypic frequencies of ABO blood group within studied population

		Study Populations Category		
		Case freq(N)	Population freq(N)	Control freq(N)
Genotypes Of Study Populations	AA	0.0361 (4)	0.034225(574)	0.04(4)
	AO	0.2508 (28)	0.24716(3841)	0.268(29)
	AB	0.057 (5)	0.05476(381)	0.052(6)
	BB	0.0225 (3)	0.021904(352)	0.0169(2)
	BO	0.198 (22)	0.197728(3170)	0.1742(18)
	OO	0.4356 (47)	0.446224(5969)	0.4489(49)
Total		1.00 (109)	1.00 (14287)	1.00 (108)

Table 16 shows the comparison of leprosy patients' ABO blood group phenotypic frequencies pattern with the combined data of controls and population. The tabular analysis revealed that there is no significant difference in the frequency distribution of ABO blood group between leprosy patients and the combined data (2-sided Pearson chi-square; $X^2_{(3)}$ =1.694, p=0.638).

Table 16: Cross tabular comparison of Leprosy patients and combined data of controls and population

			ABO Blood Group				Total
			A	B	AB	O	
Group	Case	N	32_a	25_a	5_a	47_a	109
		Expected N	33.7	26.8	2.9	45.6	109
		% within ABO Blood Group	29.4	22.9	4.6	43.1	100
		% within Group	0.7	0.7	1.3	0.8	0.8
		% of **Total**	0.2	0.2	0.0	0.3	0.8
	Control plus Population	N	4448_a	3542_a	387_a	6018_a	14395
		Expected N	4446.3	3540.2	389.1	6019.4	14395
		% within ABO Blood Group	30.9	24.6	2.7	41.8	100
		% within Group	99.3	99.3	98.7	99.2	99.2
		% of **Total**	30.7	24.4	2.7	41.5	99.2
Total		N	4480	3567	392	6065	14504
		Expected N	4480	3567	392	6065	14504
		% within ABO Blood Group	30.9	24.6	2.7	41.8	100
		% within Group	100	100	100	100	100
		% of **Total**	30.9	24.6	2.7	41.8	100

Each subscript letter denotes a subset of Phenotype categories whose column proportions do not differ significantly from each other at the 0.05 level.

The pattern of ABO blood group phenotype distribution among the four spectrums of leprosy is presented in Table 17. The result showed that there was prominent dominance of "B", "AB", and "O" blood groups in BL, BB, and BT leprosy types respectively. However, the result also indicated that there was significant least incidence of BT in "B" blood group and BL in "O" blood group. Crosstab statistical analysis of leprosy types with ABO blood group distribution in population also showed significant association between ABO blood group and mid borderline leprosy (2-sided Pearson chi-square test; $X^2(3) = 10.31$, $P = 0.016$).

Table 17: Comparison of leprosy spectrum with population in relation to the ABO blood group

		Population N (%)	Leprosy Spectrum				Minimum expected count	N	X^2, P
			LL N (%)	BL N (%)	BB N (%)	BT N (%)			
ABO Blood Group	A	4448 (30.9)	9 (36)	7 (29.2)	5 (20.8)	7(30.4)	7	28	1.14, 0.89
	B	3542 (24.6)	6 (24)	10 (41.7)	4 (16.7)	2(8.7)	5	22	6.52, 0.16
	AB	387 (2.7)	1 (4)	1 (4.2)	3 (12.5)	0(0.0)	1	5	
	O	6018 (41.8)	9 (36)	6 (25)	12 (50)	14(60.9)	10	41	3.61, 0.46
N		14395	25	24	24	23	23	96	
X2, P			0.58, 0.902	4.67, 0.198	10.31, 0.016*	4.98, 0.173	0.33, 0.96		

******significant difference, LL= Lepromatous, BL=Borderline Lepromatous, BB= Mid-Borderline, BT= Borderline Tuberculoid*

The cross-tabular analysis of odd ratio for "A" and non-A blood types along leprosy spectrum in relation to lepromatous leprosy is indicated in Table 18. The odd of "O" blood among patients was the same as the odds of "O" among controls. Likewise, the odd of "O" among BL was the same as the odd of "O" among LL leprosy patients.

Table 18: Odd ratio comparison of ABO blood group along leprosy spectrum and population

	Case /Population			LL / BL			LL / BB			LL / BT		
	OR	95% CI	P+	OR	95% CI	P+	OR	95% CI	P+	OR	95% IC	P+
O / A	1.10	0.68–.75	0.75	0.86	0.21–3.58	1	2.40	0.60 – 9.70	0.31	2.00	0.55–7.30	0.34
B / A	1.00	0.57–1.73	0.96	2.14	0.52–8.81	0.48	1.2	0.23 – 6.39	1	0.43	0.07–2.81	0.66
AB / A	2.10	0.79–5.35	0.18	1.29	0.07–24.38	1	5.4	0.44 – 66.67	0.28	--	--	--
Non-A / A	1.10	0.70–1.69	0.82	1.37	0.41 – 4.54	0.76	2.14	0.60 – 7.69	0.35	1.29	0.39–4.30	0.77

+*Fisher's exact test p- value, OR= Odd ratio, CI= Confidential interval*

5.3. Haptoglobin concentration

Shapiro-Wilk test and an inspection of the Skewness and Kurtosis measures and standard errors as well as a visual inspections of their histogram, normal Q-Q plots, and pox plots showed that Hp plasma concentration of leprosy patients was not approximately normally distributed with mean 1.14mg/ml and standard deviation of 0.62mg/ml (P = 0.032). Likewise, the data of healthy controls were not approximately normally distributed with mean 1.30mg/ml and standard deviation of 0.75mg/ml (P = 0.007). The data were not normally distributed even after log transformation. Therefore, non-parametric test was used to analyze the homogeneity of distribution pattern and t-test was used to compare mean by gender.

Generally, Haptoglobin plasma concentration of study populations was varied with a mean 1.37mg/ml, range 8.21mg/ml, and standard deviation of 1.18mg/ml. Subjects who had a minimum detectable dose of Haptoglobin (0.078µg/ml) in their plasma accounted for about 5% (leprosy patient = 10; control = 1) of the total sample size. Likewise, 6.5% of study participants had outlier scores (greater than 3mg/ml) for plasma Hp concentration.

The amount of Haptoglobin plasma concentration of leprosy patients was not significantly differ from Haptoglobin plasma concentration of healthy controls (Mann-Whitney test, P = 0.138, cases' mean rank = 90.04, and controls' mean rank = 101.90). Similarly, based on One-way ANOVA analysis, there was no statistically significant difference among spectrums of leprosy (p = 0.70). The mean_1.37mg//ml and median_1.23mg/ml of Hp amount in type II leprosy reaction patients were greater than the mean_0.96mg/ml and median_0.69mg/ml of

Hp amount in type I leprosy reaction patients. But, there was no significant difference between the two types of reaction (independent samples t-test; $t_{(21)} = -1.254$, $p = 0.223$, 95% CI (-1.09 - 0.27)). The comparison of these two types of leprosy reactions with leprosy patient those had no reaction indicated the absence of significant difference at $\alpha = 5\%$ and 2-tailed independent samples t-test ($p > 5\%$). Table 19 depicts the comparison of Hp level in relation to leprosy patients, healthy controls, leprosy spectrum, reaction types, and bacterial index.

Table 19: Plasma Haptoglobin concentration (mg/ml) with respect to category of study participants, leprosy spectrum, leprosy reaction types, and bacterial indexes

Category			N	Mean	SD	Median	Range	p	
Category	Case	male	58	1.21	.65	1.16	2.48		
		female	37	1.03	.56	.99	2.54	0.224[a]	
		Total	95	1.14	.62	1.02	2.54		0.138[b]
	Control	male	63	1.22	.74	1.14	2.93		
		female	33	1.46	.75	1.59	2.75	0.146[a]	
		Total	97	1.32	.76	1.30	3.03		
Leprosy Spectrum	LL		23	1.22	.65	1.26	2.19		
	BL		20	1.21	.67	1.09	2.41		
	BB		23	1.00	.67	.82	2.33		
	BT		19	1.08	.49	1.02	2.15	0.700[c]	
	neural		10	1.22	.61	1.17	1.76		
	Total		95	1.14	.62	1.02	2.54		
Reaction Type	Type I		15	.96	.76	.69	2.40	0.313[c]	
	Type II		8	1.37	.73	1.23	2.06		
	No reaction		72	1.15	.58	1.03	2.43		
	Total		95	1.14	.62	1.02	2.54		
Bacterial Index	BI = 0		41	1.02	.58	.95	2.38	0.175[c]	
	BI = 1		9	1.06	.51	.99	1.70		
	BI = 2		5	1.30	.80	.99	2.08		
	BI = 3		9	1.38	.78	1.37	2.10		
	BI = 4		8	.86	.47	.91	1.51		
	BI > 4		23	1.35	.63	1.28	2.15		
	Total		95	1.14	.62	1.02	2.54		

[a]= independent sample t- test, [b]= independent sample Mann-Whitney test, [c]= One- way ANOVA test

Independent Sample t_ test analysis indicated that the distribution of Hp plasma level was not significantly differ across the category of leprosy patients by gender ($t_{(93)}$ = 1.349, P = 0.181, 95% CI [-0.083 – 0.434]). Similarly, there was no significant difference of means in healthy controls by gender ($t_{(94)}$ = -1.465, P = 0.146). Furthermore, the mean and median of Hp concentration was significantly higher in female controls (1.46mg/ml, 1.59mg/ml respectively) than in female leprosy patients (1.03mg/ml, 0.99mg/ml respectively) (Independent Sample t_ test, P = 0.009). In the same way, no significant difference between mean_1.22mg//ml and median_1.14mg/ml of male controls and mean_1.21mg/ml and median_1.16gm/ml of male leprosy patients (Independent Sample t_ test, P = 0.30). Plasma level comparison of Hp within 95% confidential interval between study populations by gender is indicated in Figure 2.

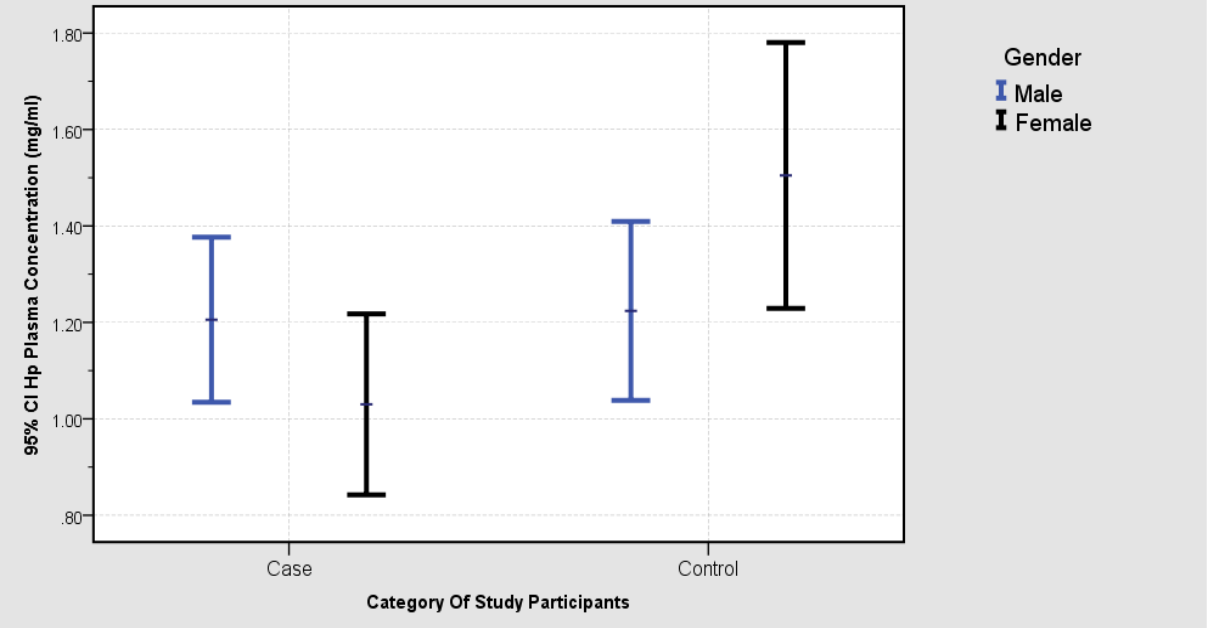


Figure 2: Error bar plot showing 95% CI comparison of Hp concentration by gender

The overall haptoglobin level varied between 0.078mg/ml to 2mg/ml within the categories of leprosy type and only 13% (14/109; LL = 4, BL = 4, BB = 3, BT = 1, TT = 0, and neural = 2) observations contained greater than 2mg/ml of Hp in their plasma. From the 87% those scored below 2mg/ml, 35% (38/109; LL = 9, BL = 7, BB = 8, BT = 10, TT = 0, and Neural = 4) had plasma Hp concentration between 1mg/ml and 2mg/ml and the rest 52% (57/109; LL = 12, BL = 13, BB = 13, BT = 12, TT = 1, Neural = 6) had less than 1mg/ml. Out of 109

leprosy patients 9.2% (10/109; LL = 2, BL = 3, BB = 0, BT = 4, TT = --, Neural = 1) had Hp concentration less than the minimum detectable amount of Hp in plasma (0.078µg/ml).

Figure 3 indicates the Hp concentration distribution along leprosy spectrum in relation to types of leprosy reaction. The mean_0.86mg/ml, median_0.74mg/ml, and range_2.22mg/ml of Hp level in LL patients with type II reaction were less compared to the mean_1.19mg/ml, median_1.27mg/ml, and range_2.24mg/ml of Hp level in LL patients who had no reaction. Similarly, the mean and median of Hp level in LL patients with type II reaction were also less than the mean_1.67mg/ml and median_1.43mg/ml of BL patients having type II reaction. The detail comparisons are indicated in Table 20.

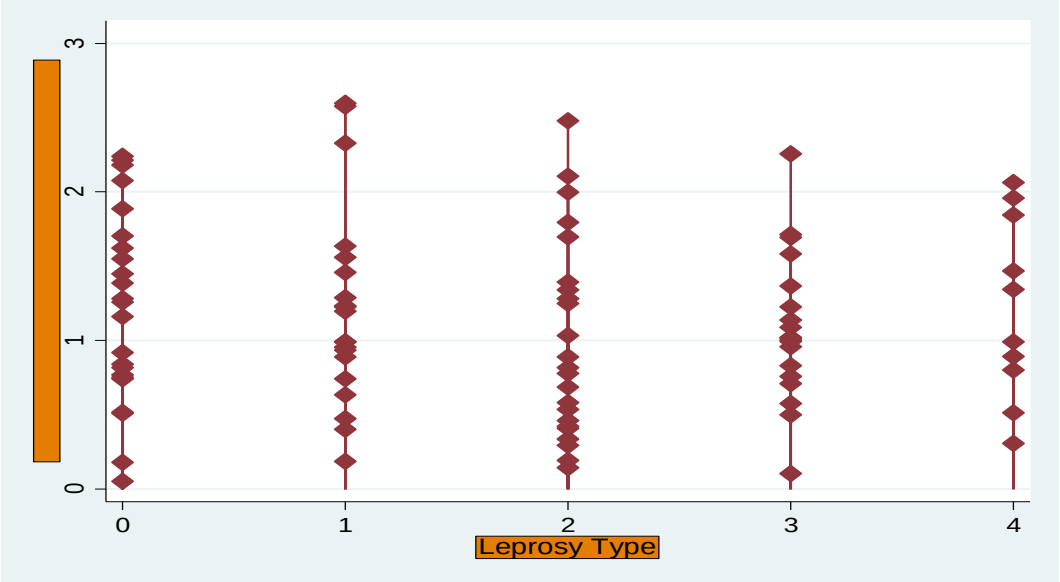


Figure 3: Comparison of plasma Haptoglobin level along leprosy spectrum, 0= Lepromatous, 1=Borderline Lepromatous, 2= Mid-Borderline, 3= Borderline Tuberculoid, 4= Neural

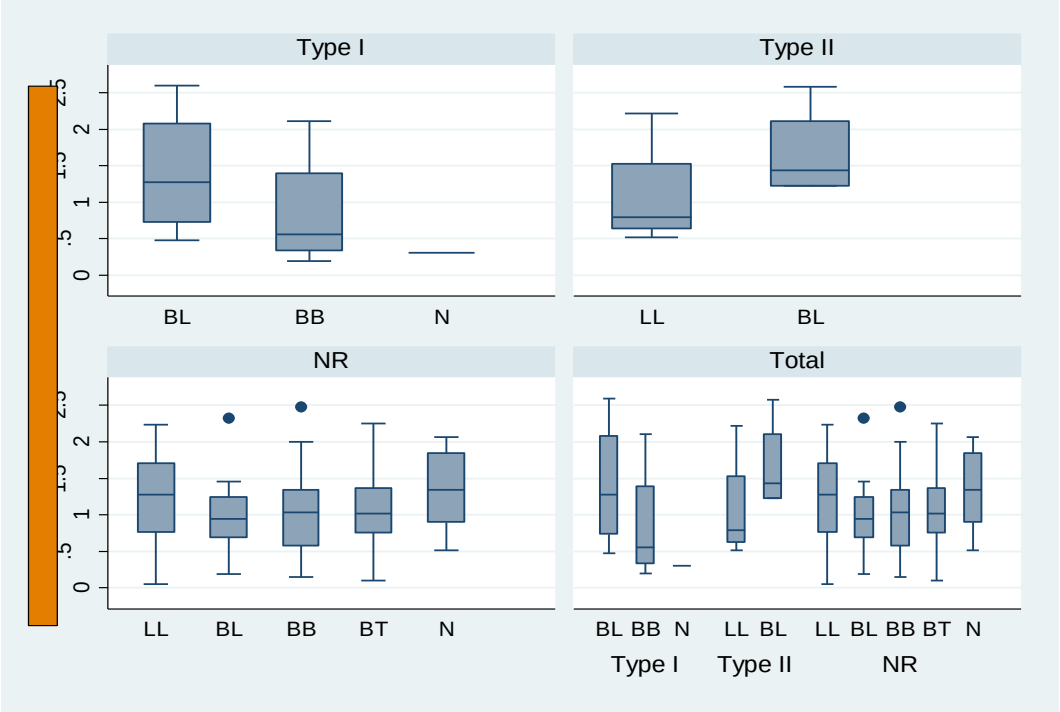


Figure 4: Plasma haptoglobin concentration comparison by leproy type and reaction
N= neural leprosy, NR= No Reaction, LL= Lepromatous, BL=Borderline Lepromatous, BB= Mid-Borderline, BT= Borderline Tuberculoid

Table 20: Plasma Haptoglobin concentration (mg/ml) by leprosy reaction type and spectrum

Leprosy Reaction Type	Ridley & Jopling Leprosy Type	Mean	N	SD	Median	Range	% of Total N
Type I	BL	1.12	5	1.01	0.99	2.60	4.6
	BB	0.85	10	0.68	0.56	1.9	9.2
	BT	0.00	1		0.00	0.00	0.9
	Neural	2.30	2	2.82	2.30	3.99	1.8
	Total	1.04	18	1.11	0.58	4.30	16.5
Type II	LL	0.86	5	0.82	0.74	2.22	4.6
	BL	1.67	4	0.64	1.43	1.35	3.7
	Total	1.22	9	0.82	1.22	2.58	8.3
NR	LL	1.19	20	0.68	1.27	2.24	18.3
	BL	1.06	15	1.00	0.93	3.94	13.8
	BB	1.27	14	0.89	1.14	3.28	12.8
	BT	0.93	22	0.60	1.00	2.26	20.2
	TT	0.28	1	.	0.28	0.00	0.9
	Neural	1.19	10	0.67	1.17	2.07	9.2
	Total	1.10	82	0.76	1.00	3.94	75.2
Total	LL	1.12	25	0.71	1.16	2.24	22.9
	BL	1.18	24	0.94	0.99	3.94	22.0
	BB	1.10	24	0.82	0.85	3.28	22.0
	BT	0.89	23	0.61	0.99	2.26	21.1
	TT	0.28	1	.	0.28	0.00	0.9
	Neural	1.37	12	1.13	1.17	4.30	11.0
	Total	1.10	109	0.82	0.99	4.30	100

NR= have no leprosy reaction, SD= standard deviation

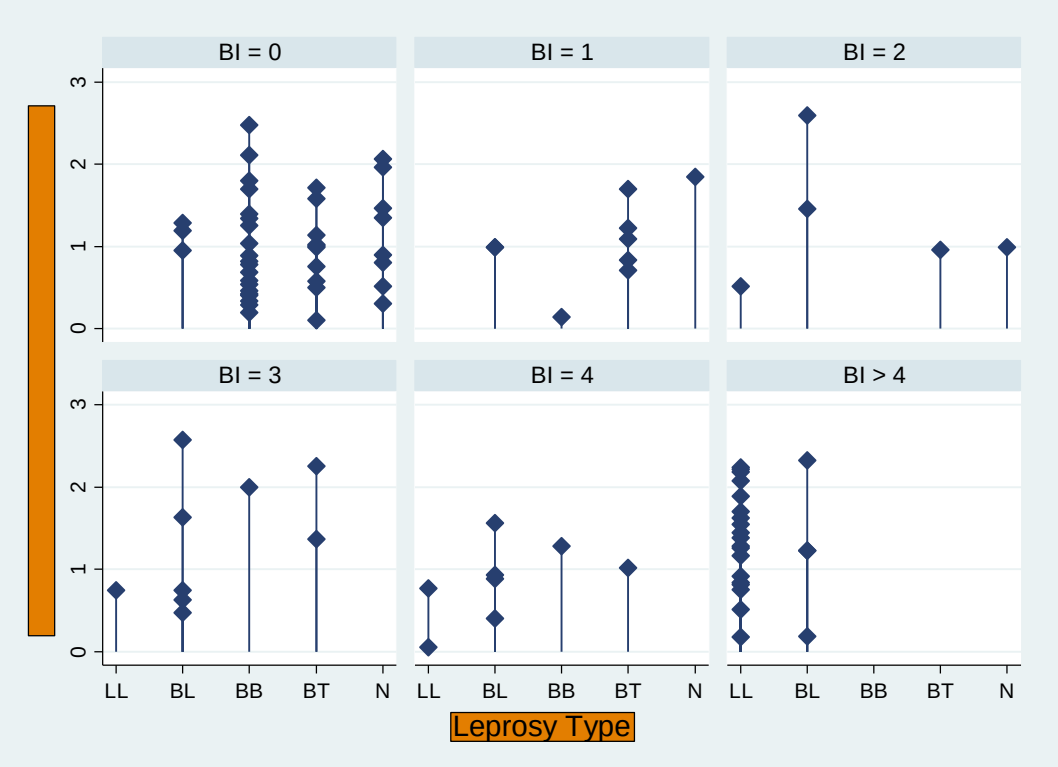


Figure 5: Plasma haptoglobin concentration comparison by leprosy type and bacterial index

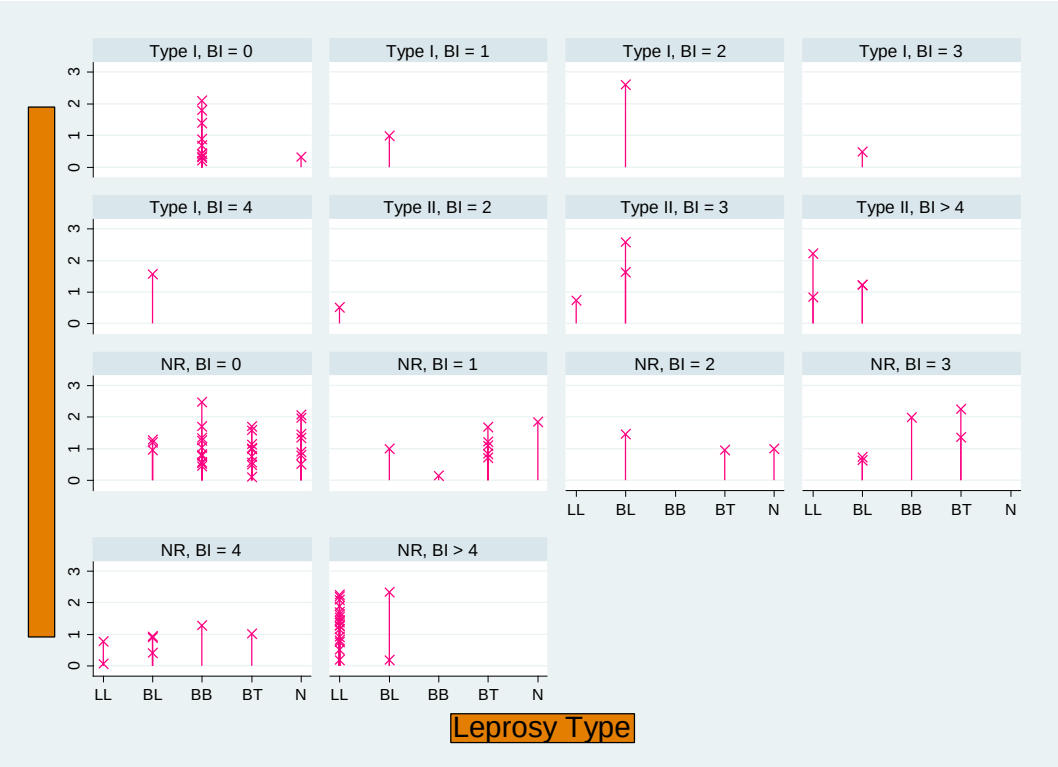


Figure 6: Plasma haptoglobin comparison by bacterial index and leprosy reaction

5.4. Haptoglobin polymorphism

The PCR reaction with A&B primer combination produced Hp1 specific 1711bp length DNA section and/or Hp2 specific 3435bp (faint band in the gel) length DNA fragment products. However, PCR reaction containing C&D primer combination produced Hp2 specific 349bp length DNA amplification product (Figure 7).

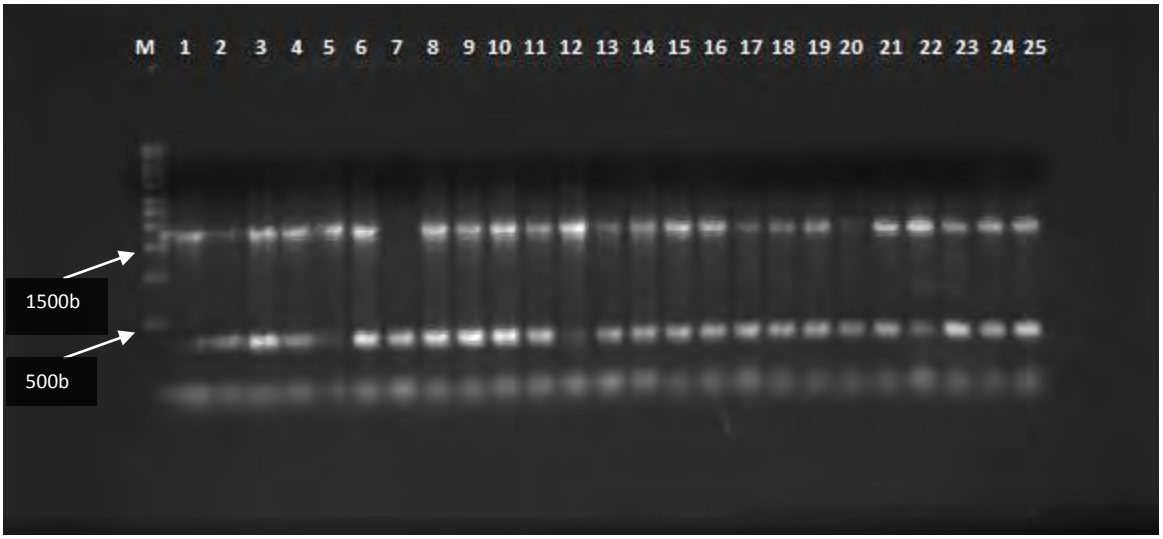


Figure 7: Gel picture of PCR amplification products, M = DNA size marker (1kb, Sigma-Aldrich), 1-25 are Hp1-2 individuals except 7 which is Hp2-2.

From a target population of 217 individuals, Hp genotype was obtained for 126 subjects with a distribution of: Hp^{1-1}_5.6% (7/126: Males 4 and Females 3), Hp^{2-2}_10.3% (13/126; Males 11 and Females 2), and Hp^{1-2}_84.1% (106/126: Males 64 and Females 42).

Table 21: Genotypic distribution pattern of Hp gene

		Hp genotype			Total	P
		Hp1-1 N(%)	Hp2-2 N(%)	Hp1-2 N(%)		
Category Of Study	Case	4 (6.35)	11(17.46)	48(76.19)	63(100)	0.019*
Participants	Control	3(4.76)	2(3.18)	58(92.06)	63(100)	
Total		7(5.56)	13(10.32)	106(84.12)	126(100)	
OR		1	4.125	0.621		

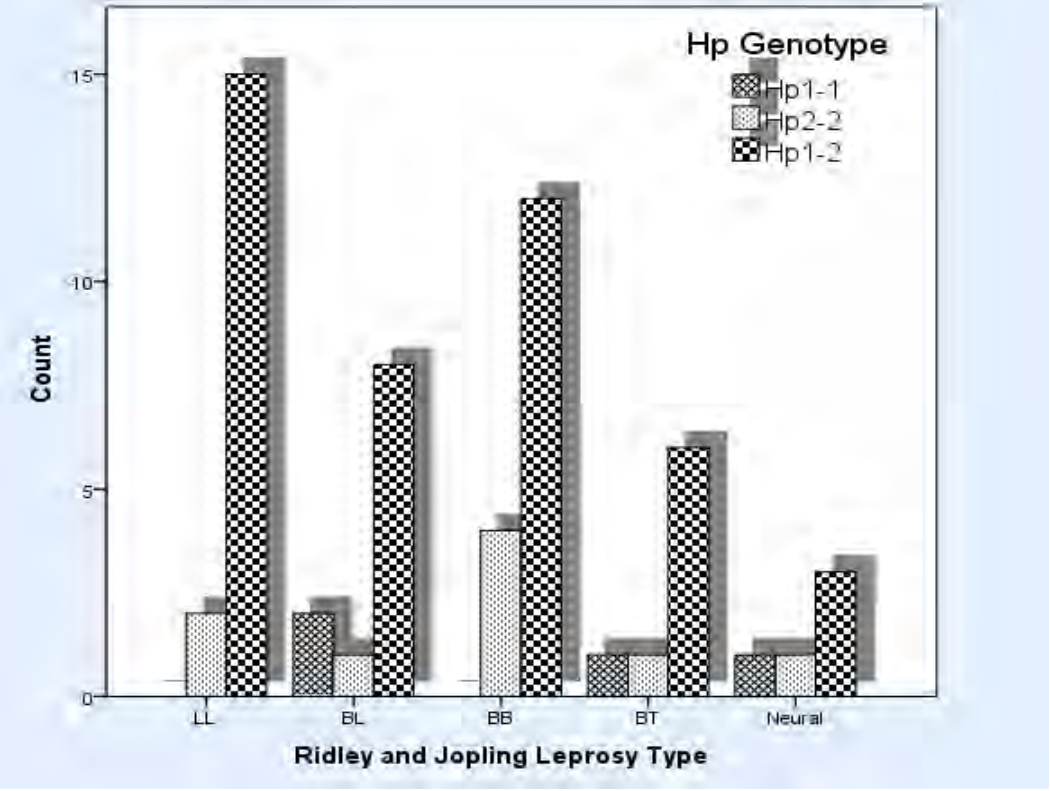
**Statistically significant: likelihood ratio test, OR= odd ratio with Hp^{1-1} as a reference genotype*

Table 22: Distribution pattern of Hp alleles

		Hp allele		Total	P
		Hp1 N(%)	Hp2 N(%)		
Category Of Study Participants	Case	56(44.44)	70(55.56)	126	0.313+
	Control	64(50.79)	62(49.21)	126	
Total		120	132	252	
OR		0.775			

+*likelihood ratio test, OR_ odd ratio*

There was a significant difference in genotype distribution between patients and controls (P = 0.019). HP^{2-2} was the most frequent genotype in leprosy patients compared to its frequency in healthy controls (17.46% and 3.17%, respectively). Data illustrated in Table 21. For leprosy, the Hp^1 allele frequency was 44% and for Hp^2 allele was 56%: while for control subjects it was 51% and 49% for Hp^1 and Hp^2 alleles, respectively. Hp^2 allele frequency was higher in leprosy patients, although this difference lacked significance (P = 0.313) (Table 22). The distribution of Haptoglobin genotype along leprosy spectrum is presented in Figure 8.



`Figure 8: Haptoglobin genotype distribution along leprosy spectrum, LL= Lepromatous, BL=Borderline Lepromatous, BB= Mid-Borderline, BT= Borderline Tuberculoid

Allele frequency for Hp^1 and Hp^2 of each 50.00% in BL and neural leprosy were similar to the 50.79% and 49.21% found in the healthy controls. Likewise, Hp^2 frequencies of 55.88%, 61.77% and 54.55% in the LL, BB and BT, respectively, were not significantly different from 49.21% found in healthy controls (Figure 9).

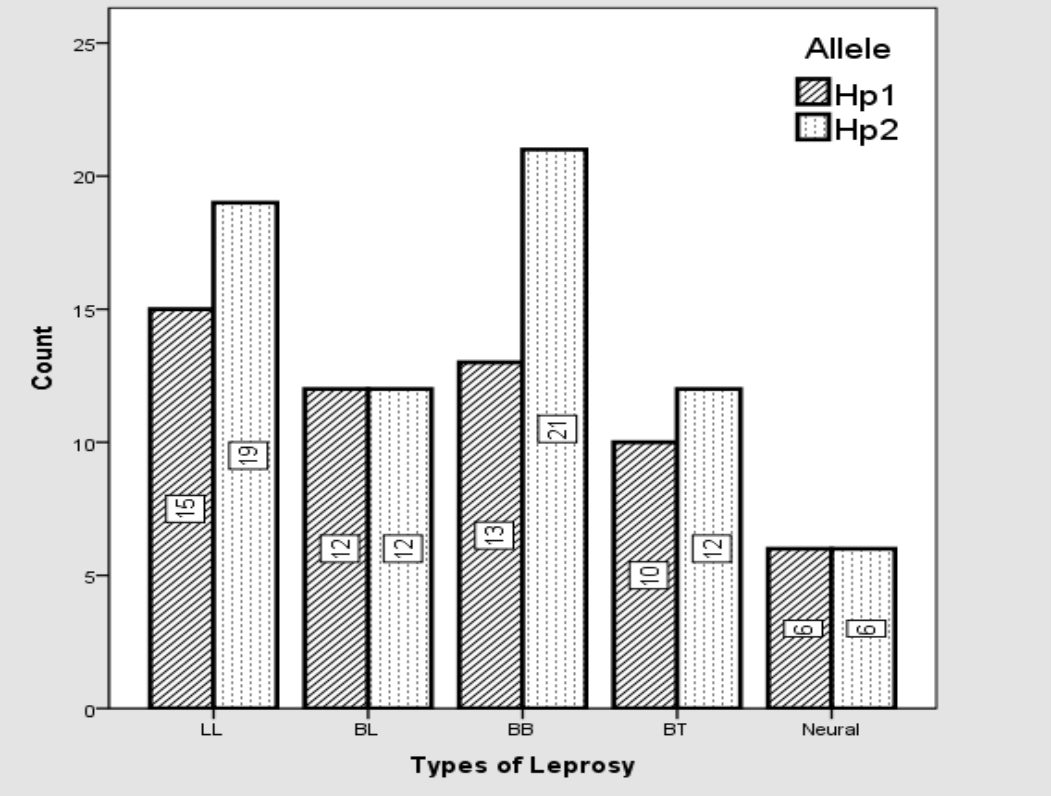


Figure 9: Hp allele distribution along leprosy spectrum LL= Lepromatous, BL=Borderline Lepromatous, BB= Mid-Borderline, BT= Borderline Tuberculoid

Table-23 shows the relationship between Hp phenotypes/genotypes and their corresponding plasma level in both patients and healthy controls. This study, there was no significant differences between the Hp phenotypes and Hp concentration ($p > 0.05$).

Table 23: Comparison of plasma Hp concentration by genotype and category of study participants

Genotype	Category Of Study Participants	Mean	N	SD	Median	Range	P
Hp1-1	Case	0.96	4	0.24	1.01	0.56	
	Control	1.12	3	0.32	1.30	0.55	0.472
	Total	1.03	7	0.26	1.02	0.68	
Hp2-2	Case	1.27	9	0.65	1.23	2.29	
	Control	1.72	2	0.58	1.72	0.82	0.386
	Total	1.35	11	0.63	1.32	2.29	
Hp1-2	Case	1.21	44	0.66	1.06	2.54	
	Control	1.40	51	0.74	1.35	2.78	0.191
	Total	1.31	95	0.71	1.28	2.92	
Total	Case	1.20	57	0.63	1.09	2.54	
	Control	1.40	56	0.72	1.32	2.78	
	Total	1.30	113	0.68	1.26	2.92	

6. Discussion

6.1. ABO blood group

In this study, high percentage of "O" blood group (43.1%) phenotype was observed among leprosy patients followed by A_29.4%, B_22.9%, and AB_4.6% that nearly concordant with the official data from Ethiopian National Red Cross. According to data of blood donors at Ethiopian National Red Cross, blood group "O" predominates (41.8%) in the population, followed by group A_30.9%, B_24.7% and AB_2.7% (Haftom, 2011).

The results of this study revealed that ABO blood group distribution pattern in leprosy patients is similar to the distribution pattern of ABO blood group in the total population. This may suggest that there is no significant association between ABO blood group and prevalence of leprosy in general. On the phenotypic frequencies of ABO blood group, our result is consistent with previous other findings. For instance, similar study in China among 459 Chinese patients of leprosy and 15,261 non-leprosy individuals revealed the percentage frequencies of the ABO blood group as 26%, 27.9%, 5.4%, and 40.5% in leprosy patients and 26%, 25%, 5.5%, and 43.5% in healthy controls for A, B, AB, and O respectively (Saha et al., 1971). Similarly, a study conducted on 100 clinically/bacteriologically diagnosed Bangladeshis leprosy patients was come up with A_30%, B_32%, AB_8%, and O_30% in leprosy patients and A_24.17%, B_35.54%, AB_8.27%, and O_33.05% in the population. The analysis of combined data of 27 series from 14 different countries on leprosy association with ABO blood group among 11,261leprosy patients and 390,602 healthy controls revealed similar conclusion (Salzano, 1967). However, another analysis of 31 series having 12,299 leprosy patients and 393,023 healthy controls indicated high frequency of "B" and low frequency of "O" in leprosy patient with non-significant difference (Vogel, 1968). Table 24 compares the present study with previous three studies in China, Bangladesh, and India (Ahamad et al., 2008; Saha et al., 1971; Singh and Ojha, 1967).

Table 24: Comparison of ABO blood group among leprosy patients in different studies/countries

Author	Country		Blood type			
			A%	B%	AB%	O%
Singh and Ojha, 1967	India	Case	30.49	27.17	8.84	33.5
		Control	24.74	33.26	8.2	33.8
Saha et al., 1971	China	Case	26.1	27.9	5.4	40.5
		Control	26	25	5.5	43.5
Ahamad et al., 2008	Bangladesh	Case	30	32	8	30
		Control	24.17	35.54	8.27	33.05
Present study	Ethiopia	Case	29.4	22.9	4.6	43.1
		Control	30.9	24.6	2.7	41.8

Although the sample size is small, in this study, high proportion of individuals with blood group O_60.9%, B_41.7%, and AB_8.7% were found in BT, BL, and BB leprosy type patients as compared to the distribution in the total population O_41.8%, B_24.6%, and AB_2.7%. From this it might be possible to suggest that individuals with O, B, and AB blood types are more likely to develop BT, BL, and BB leprosy types, respectively.

Similarly, the proportion of blood group "B" was significantly increasing along leprosy spectrum [BT (8.7%) > BB (16.7%) > BL (41.7%)], which might suggest the association

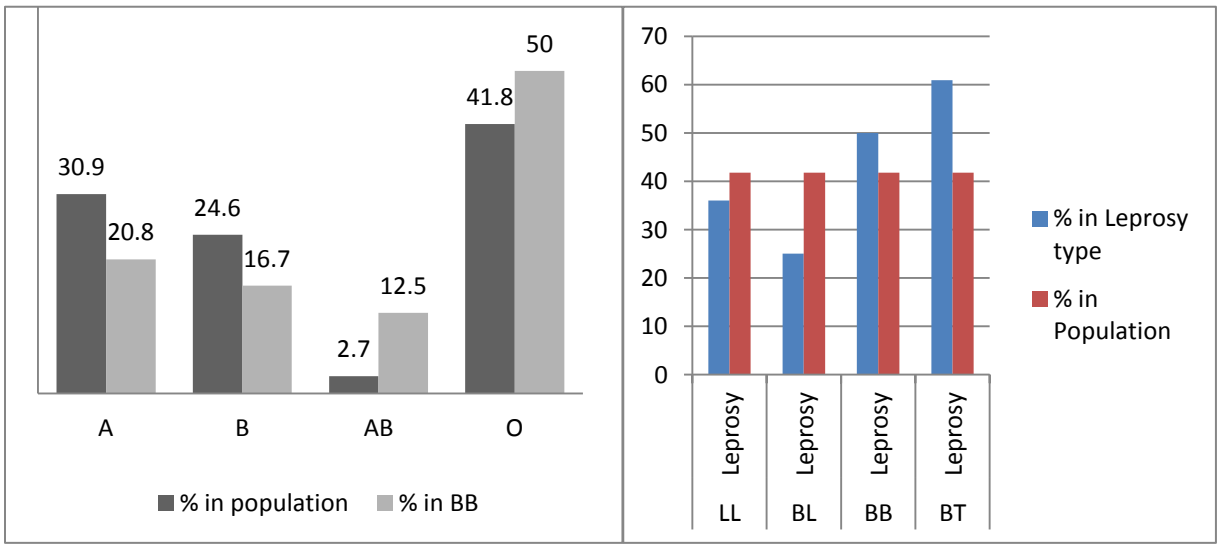


Figure 10: A, distribution of ABO blood group in BB: B, frequency of O blood type along leprosy spectrum

between “B” blood group and immunological transition along leprosy spectrum. Our study also indicates that the distributions of “A”, “B”, “AB”, and “O” in BB leprosy type were not concordance with the “A”, “B”, “AB”, and “O” blood groups in the total population.

In this study, the expected frequency genotype in leprosy patient AA_3.61%, AO_25.08%, AB_5.70%, BB_2.25%, BO_19.80%, OO_43.56% is not significantly differ from the expected genotypic frequencies of the population AA_3.42%, AO_24.72%, AB_5.48%, BB_2.19%, BO_19.77%, OO_44.62%. In the same way, this study also suggests that there is no significant association between the three alleles (i^A i^B i^O) of ABO blood group and leprosy as compared to population’s allele frequency.

6.2. Haptoglobin plasma concentration

The results obtained from this study demonstrate that there is no significant difference between plasma haptoglobin concentrations of leprosy patients (1.14mg/ml) and plasma haptoglobin concentration of healthy individuals (1.32mg/ml). In this case-control study, the concentration of haptoglobin in male leprosy patients (1.21mg/ml) is similar to the concentration of haptoglobin in male healthy controls (1.22mg/ml), whereas the plasma haptoglobin in female leprosy patients is significantly greater than the amount of plasma Hp in female healthy controls. The mean values of plasma haptoglobin concentrations obtained from different laboratories show significant differences (0.5 g/L to 1.5 g/L, 0.75 g/L to 1.75 g/L, 0.7 g/L to 1.3 g/L, 0.5 g/L to 2.2g/L) (Dobryszycka, 1997), thus, 0.3 to 3g/L has been taking as a reference range in healthy adults (Asleh and Levy, 2005; Langlois and Delanghe, 1996a). The results of our study fall within the range of plasma haptoglobin level in normal adult humans. Although there is no diurnal variation of an individual plasma haptoglobin level, there is slight variation from person to person. Unlike our results, many studies on the association of haptoglobin with infectious diseases and inflammation demonstrated haptoglobin concentration elevation during infections and inflammations, tissue destruction, and various malignant diseases (Rougemont et al., 1980; Thomsen et al., 2013).

Though we couldn’t get related literatures on the examination of haptoglobin concentration along leprosy spectrum, the concentration of plasma haptoglobin in lepromatous leprosy patients of this study was compared to the plasma haptoglobin concentration in untreated LL leprosy patients from Sritharan and colleague studies to determine dapsone induction of low

plasma haptoglobin concentration among 22 untreated lepromatous leprosy patient and 11 controls (Sritharan et al., 1981). The comparison showed slightly similar but the two studies differ by lab method and by the studied gender.

This study does not reveal statistically significant relationships between the plasma haptoglobin concentration and leprosy spectrum. In the same way, there is no statistical significant difference between the two types of leprosy reaction. However, there is an increment of plasma haptoglobin level across leprosy spectrum and along the severity of the disease. This suggests the possibility of down-regulatory or up-regulatory effects of leprosy patient's immune factors [particularly IL-6] on the expression of Hp gene (Oliviero and Riccardo, 1989).

The increment or decrement of plasma haptoglobin concentration directly depends on the rate of Hp gene expression and the rate at which the protein is released from stored granules in activated neutrophils. Haptoglobin gene expression is triggered by anti-inflammatory cytokines particularly IL-6 and IL-1(Oliviero and Riccardo, 1989). Any factor that influences the expression of IL6 or IL-1 genes has declining or elevation effect on plasma haptoglobin level. Th2 cells are characterized by their secretion of IL-4, IL-5, IL-6, IL-10, and IL-13 which promote Th2-dominant immune response to *M. leprae* indicating severe poles of leprosy development (Pinheiro et al., 2011; Stefani et al., 2003). In contrast, Th1 subtype is characterized by predominant production of pro-inflammatory cytokines like interferon γ (IFN-γ), tumour necrosis factor-beta (TNF-β), and interleukin 2 (IL-2) which promote Th1-dominant immune response to *M. leprae* indicating the development of a milder poles of leprosy (Misra et al., 1995; Stefani et al., 2003). On the other hand, IFN-gamma inhibits the proliferation of Th2 cells, and increased concentration of IL-4 and IL-6 prevent the generation of Th1 from naïve T-cell (Kidd, 2003). Additionally, resent analysis of serum level of cytokines in leprosy patients indicated significant rise of IL-10 and IL-1β cytokines in multibacillary patients(Madan et al., 2011). Similar study also indicated correlation between plasma level of IL-6 and the rise in the expression of IL6 mRNAs in ENL lesions (Jarduli et al., 2013; Madan et al., 2011).

Therefore, considering the strong relationships between leprosy immunology and Hp gene regulatory mechanisms, it may be possible to suggest that: first, the relative increment of

plasma haptoglobin concentration with the severity of leprosy is may be an indication for the increment of Hp gene expression rate with the severity of leprosy. Second, the relative increment of haptoglobin level with leprosy severity is may be an indication for the shift of leprosy patient's immune response or arise of more severe form of leprosy (Figure 11).

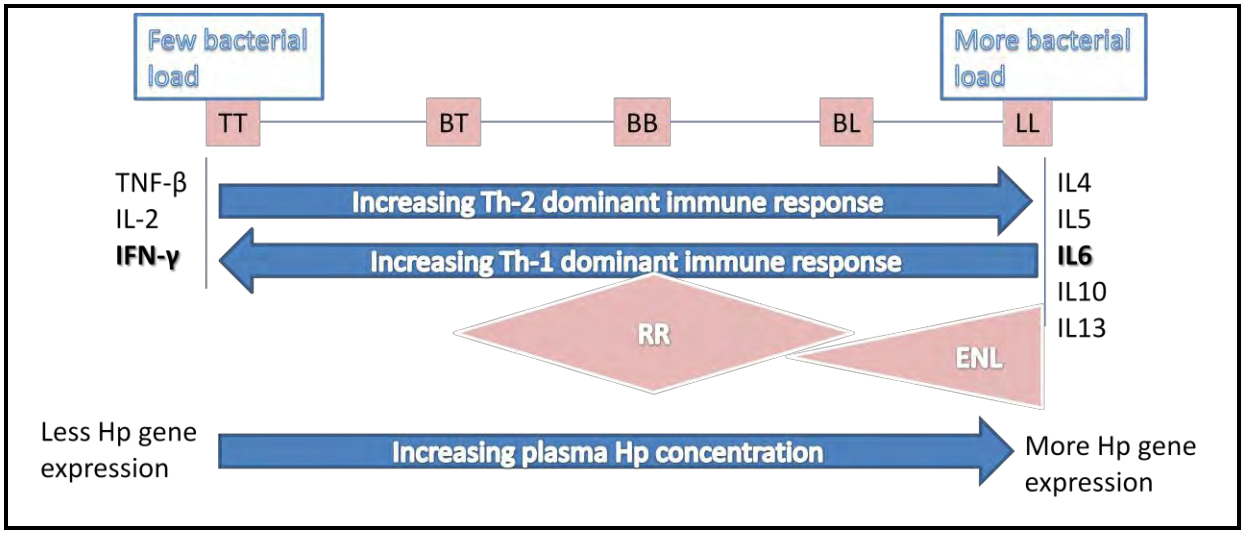


Figure 11: Hp gene expression relation with some leprosy features

6.3. Haptoglobin polymorphism

Many biological functions of haptoglobin depend on genotypes. For example, Hp1-1 individuals have greater hemoglobin binding capacity compared to individuals with Hp1-2 and Hp2-2 phenotypes. Hp2-2-Hb complex has the least CD163 binding affinity (Nielsen and Moestrup, 2009) as a result, the clearance of hemoglobin from circulation is less effective in Hp2-2 individuals. The reason of this is attributed to the larger molecular size of α2 subunit (Gueye et al., 2006; Langlois and Delanghe, 1996a). Therefore, Hp 1-1 phenotype is able to bind more hemoglobin on a molar basis than the two Hp phenotypes. Similarly, haptoglobin molecules in individuals with Hp1-1 phenotype are also more efficient antioxidants, since the smaller size of Hp1-1 facilitates its entry to extra-vascular sites of oxidative tissue injury compared to products of the Hp2 allele (Melamed-Frank et al., 2001). In another way, there is a significantly greater glomerular sieving of haptoglobin in subjects with Hp1-1 phenotype.

Additionally, the oxidative protection of Hp1 is more effective compared to Hp2 allele (Moreira et al., 2009) thus, the anti-oxidant activity of haptoglobin is phenotype dependent. Hp1-1 is the most active antioxidant phenotype and Hp2-2 has the least active antioxidant property (Melamed-Frank et al., 2001), and then Hp2-1 being intermediate phenotype (Gueye et al., 2006).

Furthermore, haptoglobin regulates the balance of Th1and Th2 by cytokine production from macrophages exposed to Hp-Hb complex via CD163 dependent mechanism (Guetta et al., 2007; Wobeto et al., 2008). For example, the Hp1-1–Hb complex promotes the secretion of more IL-6 and IL-10 than the Hp2-2–Hb complex (Guetta et al., 2007) and hence, increases Th2 dependent immune response.

This study demonstrated that the frequency pattern of Hp genotype is significantly different from healthy controls. This result is slightly in agreement with the findings of Ananthakrishnan and colleague who reported an association of haptoglobin with leprosy in Angola among 905 leprosy patients and healthy controls (Ananthakrishnan et al., 1973). However, our result is not consistent with the study conducted among 80 leprosy patient and 100 healthy controls in India by Saoji and colleagues who found non-significant relationship between Hp genotypic distribution and leprosy (Saoji et al., 1980). Table 25 illustrates the comparison genotypic distribution of present study with other three studies in Africa [South Africa, Angola, and Mozambique] (Hitzeroth et al., 1978). The trend in this study shows a lower frequency of leprosy patients with the Hp1-2 phenotype and higher frequency of leprosy patients with Hp2-2 than in healthy controls with the Hp1-2 and Hp2-2 phenotypes, respectively. Similar study in South Africa showed Hp2-2_30.9% and Hp1-2_38.2% in leprosy patients compared to Hp2-2_25.8% and Hp1-2_39.2% in healthy controls (Hitzeroth et al., 1978). However, a data from India showed relatively low frequency of Hp2-2_ 65% in leprosy patients as compared to Hp2-2_71% in healthy controls (Saoji et al., 1980).
The data of this study also showed absence of statistically significant association between Hp alleles and leprosy.

Table 25: Comparison of Hp polymorphism among leprosy patients in four studies in Africa

Country		Hp11%	Hp12%	Hp22%	Hp1%	Hp2%
Angola	Case	48.90	44.20	6.90	71.00	29.00
	Control	53.90	35.50	11.00	71.30	28.70
Mozambique	Case	40.80	47.20	12.00	66.40	35.60
	Control	37.60	42.20	20.20	58.70	41.30
South Africa	Case	22.90	38.20	30.90	50.00	50.00
	Control	27.90	39.20	25.80	51.00	49.00
Ethiopia (present study)	Case	6.35	76.19	17.46	44.44	56.56
	Control	4.76	92.06	3.18	50.79	49.21

In this study, we found 100% frequency of Hp2-2 and Hp1-2 together in LL leprosy patients. This is similar to the finding of Saoji et al who reported 92% frequency of Hp2-2 and Hp1-2 in LL leprosy patients (Saoji et al., 1980).

7. Conclusions

The results of this study showed that the distribution of ABO blood group phenotype, genotype, and alleles in leprosy patients were not significantly differing from population. However, there was a lower percentage of blood group O, A, and B in borderline lepromatous leprosy, borderline leprosy, and in borderline tuberculoid leprosy, respectively. In contrast, there was high percentage of O blood group in BB and BT leprosy patients compared to population ABO blood group distribution. There was also statistically significant association between ABO blood group distribution and borderline leprosy occurrence.

The results of this study also show that there was no statistically significant mean difference between plasma haptoglobin concentration of leprosy patients and healthy controls. However, the concentration of Haptoglobin was increasing along leprosy spectrum with non-significant difference. Similarly, the mean of plasma haptoglobin concentration was increasing with bacterial dose in leprosy patients and there was also higher plasma haptoglobin concentration in leprosy patients having type II reaction, although these differences lacked difference.

The genotypic frequency of Hp gene in leprosy patients was significantly different from Hp genotypic frequency in healthy controls and Hp2-2 was the most frequent genotype in leprosy patients compared to healthy controls. Similarly, Hp2 allele was more frequent in leprosy patients but the difference was not significant compared to its frequency in the population.

Recommendation

This study mainly focused on the two common Hp gene alleles. Additionally, the study also mainly focused on the four leprosy types, but not on other features of the disease such as leprosy reaction, *M. leprae* dose in patients, and disability grades. Therefore, we recommend for:

- Further researches, which focus on the different features of leprosy
- Study on single nucleotide polymorphism of Hp gene among leprosy patients
- More research activities on Hp gene polymorphism in Ethiopia to enrich data of Hp gene as a biomarker
- Detailed further multiplex PCR study of Hp gene among leprosy patients

References

Adib-Conquy, M., Scott-Algara, D., Cavaillon, J.-M. and Souza-Fonseca-Guimaraes, F. (2014). TLR-mediated activation of NK cells and their role in bacterial/viral immune responses in mammals. *Immunol. Cell Biol.* **92**: 256-262.

Ahamad, S., Mirjada, R. and Pahan, D. (2008). Patttern of ABO and RH (D) blood group among leprosy patients. *JCMCTA* **19**: 51-53.

Alcais, A., Alter, A., Antoni, G., Orlova, M., Nguyen, V. T., Singh, M., Vanderborght, P. R., Katoch, K., Mira, M. T., Vu, H. T., Ngyuen, T. H., Nguyen, N. B., Moraes, M., Mehra, N., Schurr, E. and Abel, L. (2007). Stepwise replication identifies a low-producing lymphotoxin-alpha allele as a major risk factor for early-onset leprosy. *Nat. Genet.* **39**: 517-22.

An, J., Fan, Z., Zhuang, Z., Qin, Y., Gao, S., Li, J. and Wang, L. (2004). Proteomic analysis of blood level of proteins before and after operation in patients with esophageal squamous cell carcinoma at high incidence area in Henan Province. *World J. Gastroenterol.* **10**: 3365 – 3368.

Ananthakrishnan, R., Walter, H., Kellermann, G. and Matznetter, T. (1973). Further studies on associations between leprosy and genetic markers in human serum. *Humangenetik* **19**: 183-92.

Asaf, R., Blum, S., Roguin, A., Litman, S., Kheir, J., Frisch, A., Lotan, R. and A., P. L. (2009). Haptoglobin genotype is a determinant of survival and cardiac remodeling after myocardial infarction in diabetic mice. *Cardiovascular Diabetol.* **29**: 2840-8.

Asleh, R. and Levy, A. (2005). In vivo and in vitro studies establishing haptoglobin as a major susceptibility gene for diabetic vascular disease. *Vascular Health and Risk Management* **1**: 19–28.

Azarov, I., He, X., Jeffers, A., Basu, S., Ucer, B., Hantgan, R., Levy, H. and Kim-Shapiro, D. (2008). Rate of nitric oxide scavenging by hemoglobin bound to haptoglobin. *Nitric Oxide* **18**: 296–302.

Barker, L. (2006). *Mycobacterium leprae* interactions with the host cell: recent advances. *Indian J. Med. Res.* **123**: 748-59.

Benkmann, H., Hanssen, H., Ovenbeck, R. and Goedde, H. (1987). Distribution of alpha-1-antitrypsin and haptoglobin phenotypes in bladder cancer patients. *Hum. Hered.* **37**: 290 – 293.

Berkova, N., Gilbert, C., Goupil, S., Yan, J., Korobko, V. and Naccache, P. H. (1999). TNF-Induced Haptoglobin Release from Human Neutrophils: Pivotal Role of the TNF p55 Receptor. *J. Immunol.* **162**: 6226-6232.

Black, J. and Dixon, G. (1968). Amino-acid sequence of alpha chains of human haptoglobins. *Nature* **218**: 738-741.

Boretti, F. S., Paul, W. B., Felice, D. A., Katharina, K., Tony, G., 1, Omer, I. B., Yiping, J., Jeroen, G., Claudia, P. P., Marco, M., Gabriele, S., Abdu, I. A. and Dominik, J. S. (2009). Sequestration of extracellular hemoglobin within a haptoglobin complex decreases its hypertensive and oxidative effects in dogs and guinea pigs. *J. Clin. Investigation* **119**: 2271–2280.

Bredt, D. (1999). Endogenous nitric oxide synthesis: biological functions and pathophysiology. *Free Radic. Res.* **31**: 577-96.

Cardoso, C. C., Pereira, A. C., Brito-de-Souza, V. N., Duraes, S. M. B., Ribeiro-Alves, M., Augusto C. Nery, J., Francio, Â. S., Vanderborght, P. R., Parelli, F. P. C., Alter, A., Salgado, J. L., Sampaio, E. P., Santos, A. R., Leide WR Oliveira, M., Sarno, E. N., Schurr, E., Mira, M. T., Pacheco, A. G. and Moraes, M. O. (2011). TNF -308G>A single nucleotide polymorphism is associated with leprosy among Brazilians: A genetic epidemiology assessment, meta-analysis, and functional study. *J. Infect. Dis.* **204**: 1256-1263.

Cardoso, C. C., Pereira, A. C., de Sales Marques, C. and Moraes, M. O. (2011). Leprosy susceptibility: genetic variations regulate innate and adaptive immunity, and disease outcome. *Future Microbiol.* **6**: 533-549.

Carter, K. and Worwood, M. (2007). Haptoglobin: a review of the major allele frequencies worldwide and their association with diseases. *Int. J. Lab Hematol.* **29**: 92-110.

Chakravartti, M. and Vogel, F. (1973). A twin study on leprosy. *Top Hum. Genet.* **1**: 1-123.

Classen, A., Lloberas, J. and Celada, A. (2009). Macrophage activation: classical versus alternative. *Methods Mol. Biol.* **531**: 29-43.

Cuevas-Santos, J., Contreras, F. and McNutt, N. S. (1998). Multibacillary leprosy: lesions with macrophages positive for S100 protein and dendritic cells positive for Factor 13. *J. Cutaneous Pathol.* **25**: 530-537.

De Kleijn, D. P. V., Smeets, M. B., Kemmeren, P. P. C. W., Lim, S. k., Van Middelaar, B. J., Velema, E., Schoneveld, A., Pasterkamp, G. and Borst, C. (2002). Acute-phase protein haptoglobin is a cell migration factor involved in arterial restructuring. *FASEB J.* **16**: 1123-1125.

De la Barrera, S., Finiasz, M., Fink, S., Ilarregui, J., Aleman, M., Olivares, L., Franco, M. C., Pizzariello, G. and del Carmen Sasiain, M. (2004). NK cells modulate the cytotoxic activity generated by *Mycobacterium leprae*-hsp65 in leprosy patients: role of IL-18 and IL-13. *Clin. Exp. Immunol.* **135**: 105-13.

Delanghe, J., Langlois, M. and De Buyzere, M. (1998). Congenital anhaptoglobinemia versus acquired Hypohaptoglobinemia. *Blood* **91**: 3524.

Dempster, A., Laird, N. and Rubin, D. (1977). Maximum likelihood from incomplete data. *J. Royal Stat. Soc. Ser. B* **39**: 38.

Dobryszycka, W. (1997). Biological functions of haptoglobin - new pieces to an old puzzle. *Eur. J. Clin. Chem. Clin. Biochem.* **35**: 647-65.

El Ghmati, S. M., Van Hoeyveld, E. M., Van Strijp, J. G., Ceuppens, J. L. and Stevens, E. A. (1996). Identification of haptoglobin as an alternative ligand for CD11b/CD18. *J. Immunol.* **156**: 2542-52.

Etzioni, R., Pepe, M., Longton, G., Hu, C. and Goodman, G. (1999). Incorporating the time dimension in receiver operating characteristic curves: a case study of prostate cancer. *Medical Decision Making* **19**: 242–251.

Fava, V. and Mira, M. (2012). Genetics of leprosy. *In* "Leprosy" (E. Nunzi and C. Massone, eds.), pp. 19-26. Springer Milan.

Fitness, J., Tosh, K. and Hill, A. (2002). Genetics of susceptibility to leprosy. *Genes Immun.* **3**: 441–453.

Fowkes, F. J., Imrie, H., Migot-Nabias, F., Michon, P., Justice, A., Deloron, P., Luty, A. J. and Day, K. P. (2006). Association of haptoglobin levels with age, parasite density, and haptoglobin genotype in a malaria-endemic area of Gabon. *Am. J. Trop. Med. Hyg.* **74**: 26-30.

Frodsham, A. J. and Hill, A. V. (2004). Genetics of infectious diseases. *Hum. Mol. Genet.* **13 (2)**: R187-94.

Fujiwara, N. and Kobayashi, K. (2005). Macrophages in inflammation. *Curr. Drug Targets Inflamm. Allergy* **4**: 281-6.

Gauderman, J. and Morrison, J. (2007). Sample Size Calculation in Genetics Studies. *In* "StatNews", Vol. 71. Cornell University Statistical Consulting Unit.

Gordon, S. (2003). Alternative activation of macrophages. *Nat. Rev. Immunol.* **3**: 23-35.

Guetta, J., Strauss, M., Levy, N. S., Fahoum, L. and Levy, A. (2007). Haptoglobin genotype modulates the balance of Th1/Th2 cytokines produced by macrophages exposed to free hemoglobin. *Atherosclerosis* **191**: 48-53.

Gueye, P., Glasser, N., Ferard, G. and Lessinger, J. (2006). Influence of human haptoglobin polymorphism on oxidative stress induced by free hemoglobin on red blood cells *Clin. Chem. Lab. Med.* **44**: 542-547.

Gupta, N., Shankernarayan, N. P. and Dharmalingam, K. (2007). Serum proteome of leprosy patients undergoing erythema nodosum leprosum reaction: regulation of expression of the isoforms of haptoglobin. *J. Proteome Res.* **6**: 3669-79.

Haftom, G. (2011). Application of data mining technology in predicting the seroprevalence of HBV,HCV,HIV; the case of the National Blood Bank of Addis Ababa,Ethiopia, Addis Ababa University.

Harboe, M., Abraham, A. and Ruth, L. (2005). Challenges presented by nerve damage in leprosy. *Lepr. Rev.* **76**: 5–13.

Hedrick, P. (2005). "Genetics of Populations," 3/Ed. Jones and Bartlett Publishers, Sudbury.

Hernandez, M. O., Neves, I., Sales, J. S., Carvalho, D. S., Sarno, E. N. and Sampaio, E. P. (2003). Induction of apoptosis in monocytes by *Mycobacterium leprae* in vitro: a possible role for tumour necrosis factor-alpha. *Immunol.* **109**: 156-64.

Hitzeroth, H., Walter, H. and Hilling, M. (1978). Genetic Markers and Leprosy in South African Negroes Part I. Serum Protein Polymorphisms. *S. Afr. med. J.* **54**: 653-658.

Horwitz, M. A., Levis, W. R. and Cohn, Z. A. (1984). Defective production of monocyte-activating cytokines in lepromatous leprosy. *J. Exp. Med.* **159**: 666-78.

Huntoon, K. M., Yanping, W., Cheryl, A. E., Karen, W. B., Franklin, G. B., Protul, A. S. and Heinz, B. (2008). The acute phase protein haptoglobin regulates host immunity. *J. Leukoc. Biol.* **84**: 170-181.

Imrie, H., Ferguson, D. J. and Day, K. P. (2004). Human serum haptoglobin is toxic to *Plasmodium falciparum* in vitro. *Mol. Biochem. Parasitol.* **133**: 93-8.

Imrie, H., Fowkes, F. J., Michon, P., Tavul, L., Hume, J. C., Piper, K. P., Reeder, J. C. and Day, K. P. (2006). Haptoglobin levels are associated with haptoglobin genotype and alpha+ -Thalassemia in a malaria-endemic area. *Am. J. Trop. Med. Hyg.* **74**: 965-71.

Jarduli, L. R., Sell, A. M., Reis, P., Guimar, M., Sippert, E., Ayo, C. M., Mazini, P. S., Alves, H. V., Teixeira, J. J. V. and Visentainer, J. E. L. (2013). Role of HLA, KIR, MICA, and cytokines genes in leprosy. *Bio. Med. Res. Intern.* **2013**: 17.

Jopling, W. and Mcdougall, A. (1999). Diagnostic tests. *In* "Hand book of leprosy", pp. pp.60. CBS.

Jue, D. M., Shim, B. S. and Kang, Y. S. (1983). Inhibition of prostaglandin synthase activity of sheep seminal vesicular gland by human serum haptoglobin. *Mol. Cell Biochem.* **51**: 141-7.

Kawai, T. and Akira, S. (2006). Innate immune recognition of viral infection. *Nature Immun.* **7**: 131–7.

Khansari, N., Shakiba, Y. and Mahmoudi, M. (2009). Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. *Recent Patents on Inflamm. & Allergy Drug Discov.* **3**: 73-80.

Kidd, P. (2003). Th1/Th2 Balance: The hypothesis, its limitations, and implications for health and disease. *Altern. Med. Rev.* **8**: 223-246.

Koch, W., Wolfgang, L., Marianne, E., Ariel, R., Andrew, P. L., Albert, S. and Adnan, K. (2002). Genotyping of the common haptoglobin Hp 1/2 polymorphism based on PCR. *Clin. Chem.* **48**: 1377–1382.

Koda, Y., Soejima, M., Yoshioka, N. and Kimura, H. (1998). The haptoglobin-gene deletion responsible for anhaptoglobinemia. *Am. J. hum. genet.* **62**: 245-252.

Koda, Y., Watanabe, Y., Soejima, M., Shimada, E., Nishimura, M., Morishita, K., Moriya, S., Mitsunaga, S., Tadokoro, K. and Kimura, H. (2000). Simple PCR detection of

haptoglobin gene deletion in anhaptoglobinemic patients with antihaptoglobin antibody that causes anaphylactic transfusion reactions. *Blood* **95**: 1138-1143.

Kominato, Y., Hata, Y., Matsui, K. and Takizawa, H. (2005). Regulation of ABO gene expression. *Leg. Med.* **3**: 371-8511.

Kristiansen, M., Graversen, J. H., Jacobsen, C., Sonne, O., Hoffman, H. J., Law, S. K. and Moestrup, S. K. (2001). Identification of the haemoglobin scavenger receptor. *Nature* **409**: 198-201.

Krutzik, S. R., Ochoa, M. T., Sieling, P. A., Uematsu, S., Ng, Y. W., Legaspi, A., Liu, P. T., Cole, S. T., Godowski, P. J., Maeda, Y., Sarno, E. N., Norgard, M. V., Brennan, P. J., Akira, S., Rea, T. H. and Modlin, R. L. (2003). Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. *Nat. Med.* **9**: 525-32.

Lai, Y. A., Lai, I. H., Tseng, C. F., Lee, J. and Mao, S. J. (2007). Evidence of tandem repeat and extra thiol-groups resulted in the polymeric formation of bovine haptoglobin: a unique structure of Hp 2-2 phenotype. *J. biochem. molec. biol.* **40**: 1028-1038.

Langlois, M. and Delanghe, J. (1996). Biological and clinical significance of haptoglobin polymorphism in humans. *Clin. Chem.* **42**: 1589 - 1600.

Langlois, M., Delanghe, J., Philippe, J., Ouyang, J., Bernard, D., De Buyzere, M., Van Nooten, G. and Leroux-Roels, G. (1997). Distribution of lymphocyte subsets in bone marrow and peripheral blood is associated with haptoglobin type. Binding of haptoglobin to the B-cell lectin CD22. *Eur. J. Clin. Chem. Clin. Biochem.* **35**: 199-205.

Levy, A. P., Levy, J. E., Kalet-Litman, S., Miller-Lotan, R., Levy, N. S., Asaf, R., Guetta, J., Yang, C., Purushothaman, K. R., Fuster, V. and Moreno, P. R. (2007). Haptoglobin genotype is a determinant of iron, lipid peroxidation, and macrophage accumulation in the atherosclerotic plaque. *Arteriosclerosis, Thrombosis, and Vascular Biol.* **27**: 134-140.

Ma´rquez, L., Shen, C., Cleynen, I., Hertogh, G. D., Steen, K. V., K Machiels, Perrier, C., Ballet, V., Organe, S., Ferrante, M., Henckaerts, L., Galicia, G., Rutgeerts, P., Ceuppens, J. L. and Vermeire, S. (2011). Effects of haptoglobin polymorphisms and deficiency on susceptibility to inflammatory bowel disease and on severity of murine colitis. *Gut* **2011**.

Madan, N., K iran, A. and Ram, C. (2011). Serum cytokine profile in leprosy and its correlation with clinico-histopathological profile. *Lepr. Rev.* 371 – 382.

Maeda, N. and Smithies, O. (1986). The evolution of multigene families: Human haptoglobin genes. *Ann. Rev. Genet.* **20**: 81-108.

Maeda, N., Susan, M. M., Harris, H. F., Titus, H. J. H. and Oliver, S. (1986). Polymorphisms in the human haptoglobin gene cluster: Chromosomes with multiple haptoglobin-related (Hpr) genes. *Proc. Natl. Acad. Sci.* **83**: 7395-7399.

Maeda, Y., Mukai, T., Spencer, J. and Makino, M. (2005). Identification of an immunomodulating agent from *Mycobacterium leprae*. *Infect. Immun.* **73**: 2744-50.

Magombedze, G., Pradeep, B. J. R., Shigetoshi, E. and Vitaly, V. G. (2013). Cellular and population plasticity of helper CD4+ T-cell responses. *Front Physiol.* **4**: 1-9.

Martin, P. and Leibovich, S. (2005). Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends in Cell Biol.* **15**: 599–607.

Mathur, M., Ghimire, R., Shrestha, P. and Kedia, S. (2011). Clinicohistopathological Correlation in Leprosy. *Kathmandu Univ. Med. J.* **36**: 248-51.

Melamed-Frank, M., Lache, O., Enav, B. I., Szafranek, T., Levy, N. S., Ricklis, R. M. and Levy, A. P. (2001). Structure-function analysis of the antioxidant properties of haptoglobin. *Blood* **98**: 3693-3698.

Miller, S. (2006). Prostaglandins in health and disease: an overview. *Seminars in Arthritis and Rheumatism* **36**: 37–49.

Miller, S., Dykes, D. and Polesky, H. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl. Acids Res.* **16**: 1215.

Mira, M., Alcaïs, A. and Nguyen, V. (2004). Susceptibility to leprosy is associated with PARK2 and PACRG. *Nature* **427**: 636–640.

Miranda-Vilela, A., Akimoto, A., Alves, P., Ferreira, L., Lordelo, G., Melo, J., Grisolia, C., Oliveira, S. and Klautau-Guimarães, M. (2010). Evidence for an association between haptoglobin and MnSOD (Val9Ala) gene polymorphisms in essential hypertension based on a Brazilian case-control study. *Genet. Mol. Res.* **9**: 2166-2175.

Mircheva, G. T. (2010). Similarities and differences between haptoglobin in mammals and PIT 54 in poultry *Bulg. J. Vet. Med.* **13**: 1-12.

Misch, E. A., Berrington, W. R., Vary, J. C. and Hawn, T. R. (2010). Leprosy and the human genome. *Microbiol. and Mol. Biol. Rev.* **74**: 589-620.

Misra, N., Murtaza, A., Walker, B., Narayan, N., Misra, R., Ramesh, V., Singh, S., Colston, M. and Nath, I. (1995). Cytokine profile of circulating T cells of leprosy patients reflects both indiscriminate and polarized T-helper subsets: t-helper phenotype is stable and uninfluenced by related antigens of *Mycobacterium leprae*. *Immunol.* **86**: 97-103.

Mitchell, R., Carzino, R. and Janardhana, V. (1988). Associations between the two serum proteins haptoglobin and transferrin and leukaemia. *Hum. Hered.* **38**: 144 – 150.

Modlin, R. L. (1994). Th1-Th2 paradigm: insights from leprosy. *J. Invest. Dermatol.* **102**: 828-832.

Mohagheghpour, N., Gelber, R. H., Larrick, J. W., Sasaki, D. T., Brennan, P. J. and Engleman, E. G. (1985). Defective cell-mediated immunity in leprosy: failure of T cells from lepromatous leprosy patients to respond to *Mycobacterium leprae* is associated with defective expression of interleukin 2 receptors and is not reconstituted by interleukin 2. *J. Immunol.* **135**: 1443-9.

Moreira, L. R. S., Miranda-Vilela, A. L., Silva, I. C. R., Akimoto, A. K., Klautau-Guimarães, M. N. and Grisolia, C. K. (2009). Antioxidant effect of haptoglobin phenotypes against DNA damage induced by hydrogen peroxide in human leukocytes. *Genet. Mol. Res.* **8**: 284-290

Moreno, P., Purushothaman, K., Purushothaman, M., Muntner, P., Levy, N., Fuster, V., Fallon, J., Lento, P., Winterstern, A. and Levy, A. (2008). Haptoglobin genotype is a major determinant of the amount of iron in the human atherosclerotic plaque. *JACC* **52**: 1049–51.

Neurath, M. F., Susetta, F. and Laurie, H. G. (2003). The role of Th1/Th2 polarization in mucosal immunity. *Alter. Med. Rev.* **8**: 223-246.

Nielsen, M. J. and Moestrup, S. K. (2009). Receptor targeting of hemoglobin mediated by the haptoglobins: roles beyond heme scavenging. *Blood* **114**: 764-771.

Noordeen, S. (1994). Elimination of leprosy as a public health problem. *Indian J. Lepr.* **66**: 1-10.

Oh, S. K., Pavlotsky, N. and Tauber, A. l. (1990). Specific binding of haptoglobin to human neutrophils and its functional consequences. *J. Leukoc. Biol.* **47**: 142-148.

Oliviero, S. and Riccardo, C. (1989). The human haptoglobin gene promoter: interleukin-6-responsive elements interact with a DNA-binding protein induced by interleukin-6 *EMBO J.* **8**: 1145 - 1151.

Opal, S. M. and Vera, A. D. (2000). Anti-inflammatory cytokines. *CHEST* **117**: 1162–1172.

Otero Regino, W., Velasco, H. and Sandoval, H. (2009). Papel protector de la bilirrubina en el ser humano. *Revista Colombiana de Gastroenterologia* **24**: 293-301.

Otterbein, L. E., Bach, F. H., Alam, J., Soares, M., Tao Lu, H., Wysk, M., Davis, R. J., Flavell, R. A. and Choi, A. M. K. (2000). Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat. Med.* **6**: 422-428.

Pagano, M., Engler, R., Gelin, M. and Jayle, M. F. (1980). Kinetic study of the interaction between rat haptoglobin and rat liver cathepsin B. *Can. J. Biochem.* **58**: 410-17.

Pan, J.-P., Tsai-Mu, C., Chun-Che, S., Shu-Chiung, C., Shiu-Chin, C., Simon, J. T. and Shiau-Ting, L. (2011). Haptoglobin phenotypes and plasma haptoglobin levels in patients with abdominal aortic aneurysm. *J. Vasc. Surg.* **53**: 1189-94.

Papp, M., Foldi, I., Nemes, E., Udvardy, M., Harsfalvi, J., Altorjay, I., Mate, I., Dinya, T., Varvolgyi, C., Barta, Z., Veres, G., Lakatos, P. L., Tumpek, J., Toth, L., Szathmari, E., Kapitany, A., Gyetvai, A. and Korponay-Szabo, I. R. (2008). Haptoglobin polymorphism: A novel genetic risk factor for celiac disease development and its clinical manifestations. *Clin. Chem.* **54**: 697-704.

Pinheiro, R. O., Jorgenilce, d. S. S., Euzenir, N. S. and Elizabeth, P. S. (2011). Mycobacterium leprae–host-cell interactions and genetic determinants in leprosy: an overview. *Future Microbiol.* **6**: 217–230.

Polticelli, F., Bocedi, A., Minervini, G. and Ascenzi, P. (2008). Human haptoglobin structure and function – a molecular modelling study. *FEBS J.* **275**: 5648–5656.

Quaye, I. K. (2008). Haptoglobin, inflammation and disease. *Royal Soc. Trop. Med. and Hygiene* **102**: 735—742.

Rees, R. (1969). New prospects for the study of leprosy in the laboratory. *Bull. Wld. Hlth. Org.* **40**: 785-800.

Ridley, D. and Jopling, W. (1966). Classification of leprosy according to immunity - A five group system. *Int. J. Lepr.* **54**: 255-73.

Romagnani, S. (1991). Type 1 T helper and type 2 T helper cells: functions, regulation and role in protection and disease. *Int. J. Clin. Lab Res.* **21:** 152-8.

Romagnani, S. (1999). Th1/Th2 cells. *Inflamm. Bowel Dis.* **5**: 285-94.

Rougemont, A., Quilici, M., Delmont, J. and Ardissone, J. P. (1980). Is the HpO phenomenon in tropical populations really genetic? *Hum. Hered.* **30**: 201-3.

Sadrzadeh, H. and Bozorgmehr, J. (2004). Haptoglobin phenotypes in health and disorders. *Am. J. Clin. Pathol.* **121**: S97-S104.

Saeed, S. A., Ahmad, N. and Ahmed, S. (2007). Dual inhibition of cyclooxygenase and lipoxygenase by human haptoglobin: its polymorphism and relation to hemoglobin binding. *Biochem. Biophys. Res. Commun.* **353**: 915-20.

Saeed, S. A., Denning-Kendall, P. A., McDonald-Gibson, W. J. and Collier, H. O. J. (1981). Human haptoglobin: an endogenous inhibitor of prostaglandin synthase. *In* "Inflammation: Mechanisms and Treatment" (D. A. Willoughby and J. P. Giroud, eds.), Vol. 4, pp. 285-300. Springer Netherlands.

Saha, N., Wong, H., Banerjee, B. and Wong, M. (1971). Distribution of ABO blood groups, G6PD deficiency, and abnormal haemoglobins in leprosy. *J Med. Genet.* **315**: 315-316.

Saikawa, K. (1981). The effect of rapid socio-economic development on the frequency of leprosy in a population. *Lepr. Rev.* **52**: 167–175.

Salzano, M. (1967). Blood groups and leprosy. *J. Med. Genet.* **102**: 102-106.

Saoji, A., Jariwala, H. and Kelkar, S. (1980). Haptoglobin phenotypes in leprosy. *Int. J. Lepr. other Mycobact. Dis.* **48**: 422-5.

Schafer, F. Q. and Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.* **30**: 1191-212.

Scollard, D. (2008). The biology of nerve injury in leprosy. *Lepr. Rev.* **79**: 242–253.

Scollard, D. M., Adams, L. B., Gillis, T. P., Krahenbuhl, J. L., Truman, R. W. and Williams, D. L. (2006). The continuing challenges of leprosy. *Clin. Microbiol. Rev.* **19**: 338-381.

Singh, G. and Ojha, D. (1967). Leprosy and ABO blood groups. *J. med. Genet.* **4**: 107-108.

Snellman, O. and Sylven, B. (1967). Haptoglobin acting as a natural inhibitor of cathepsin B activity. *Nature* **216**: 1033-1033.

Spierings, E., De Boer, T., Zulianello, L. and Ottenhoff, T. H. M. (2000). Novel mechanisms in the immunopathogenesis of leprosy nerve damage: The role of schwann cells, T cells and *Mycobacterium leprae*. *Immunol. Cell Biol.* **78**: 349-355.

Sritharan, V., Bharadwaj, V., Venkatesan, K., and Girdhar, B. (1981). Dapsone induced hypohaptoglobinemia in lepromatous leprosy patients. *Int. J. Lepr. other Mycobact. Dis.* **49**: 307-310.

Stefani, M. M. A., Martelli, C. M. T. and Gillis, T. P. (2003). In situ type 1 cytokine gene expression and mechanisms associated with early leprosy progression. *J. Infec. Dis.* **188**: 1024-1031.

Suleiman, M., Aronson, D., Asleh, R., Kapeliovich, M., Roguin, A., Meisel, S., Shochat, M., Sulieman, A., Reisner, S., Markiewicz, W., Hammerman, H., Lotan, R., Levy, N. and Levy, A. (2005). Haptoglobin polymorphism predicts 30-day mortality and heart failure in patients with diabetes and acute myocardial infarction. *Diabetes* **54**: 2802-2806.

Takagi, T., Naito, Y., Uchiyama, K. and Yoshikawa, T. (2010). The role of heme oxygenase and carbon monoxide in inflammatory bowel disease. *Redox Report* **15**: 193-201.

Thomsen, J. H., Etzerodt, A., Svendsen, P. and Moestrup, S. (2013). The haptoglobin-CD163-heme oxygenase-1 pathway for hemoglobin scavenging. *Oxidative Med. and Cellular Longevity* **2013**: 11.

Tripathi, P. (2007). Nitric oxide and immune response. *Indian J. Biochem. Biophys.* **44**: 310-319.

Tsai, L. M. and Yu, D. (2014). Follicular helper T-cell memory: establishing new frontiers during antibody response. *Immunol. Cell Biol.* **92**: 57-63.

Tseng, C. F., Chia, C. L., Hsing, Y. H., Huei, C. L. and Simon, J. T. M. (2004). Antioxidant role of human haptoglobin. *Proteomics* **4**: 2221–2228.

Uyoga, S. M. (2009). Effect of Haptoglobin and Intercellullar Adhesion Molecule 1 Genotypes on the Development of Antibody Responses to *Plasmodium falciparum* Merozoite Antigens. University of Manchester.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M., Mazur, M. and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The Int. J. Biochem. & Cell Biol.* **39**, 44–84.

Vogel, F. (1968). ABO blood groups and leprosy. *J. med. Genet.* **5**: 56-57.

Wagener, F. A. D. T. G., Volk, H.-D., Willis, D., Abraham, N. G., Soares, M. P., Adema, G. J. and Figdor, C. G. (2003). Different faces of the heme-heme oxygenase system in inflammation. *Pharmacol. Rev.* **55**: 551-571.

Walker, S., and Lockwood, D. (2006). The clinical and immunological features of leprosy. *British Med. Bulletin* **78**: 103–121.

Walter, H., Bajatzadeh, M., Kellermann, G. and Matznetter, T. (1970). Association between leprosy and serum protein groups. *Hum. Genet.* **10**: 298-303.

Walter, H., Kellermann, G., Bajatzadeh, M., Kruger, J. and Chakravartti, R. (1972). Hp,Gc, Cp, Tf, Bg, and Pi phenotypes in leprosy patients and healthy control from West Benggal (India). *Hum. Genet.* **14**: 314-325.

Wejman, J. C., Hovsepian, D., Wall, J. S., Hainfeld, J. F. and Greer, J. (1984). Structure of haptoglobin and the haptoglobin-hemoglobin complex by electron microscopy. *J. Mol. Biol.* **174**: 319-41.

WHO (2013). World Health Organization: Global leprosy. *Wkly. Epidemiol. Rec.* **88**: 365–380.

Wicher, K. B. and Fries, E. (2006). Haptoglobin, a hemoglobin-binding plasma protein, is present in bony fish and mammals but not in frog and chicken. *Proceedings NASUSA* **103**: 4168-4173.

Wobeto, V. P. d. A., Tânia, R. Z. and Maria, d. F. S. (2008). Polymorphism of human haptoglobin and its clinical importance. *Genet. Mol. Biol.* **31**: 602-620.

Wunder, C. and Potter, R. F. (2003). The heme oxygenase system: its role in liver inflammation. *Curr. Drug Targets Cardiovasc. Haematol. Disord.* **3**: 199-208.

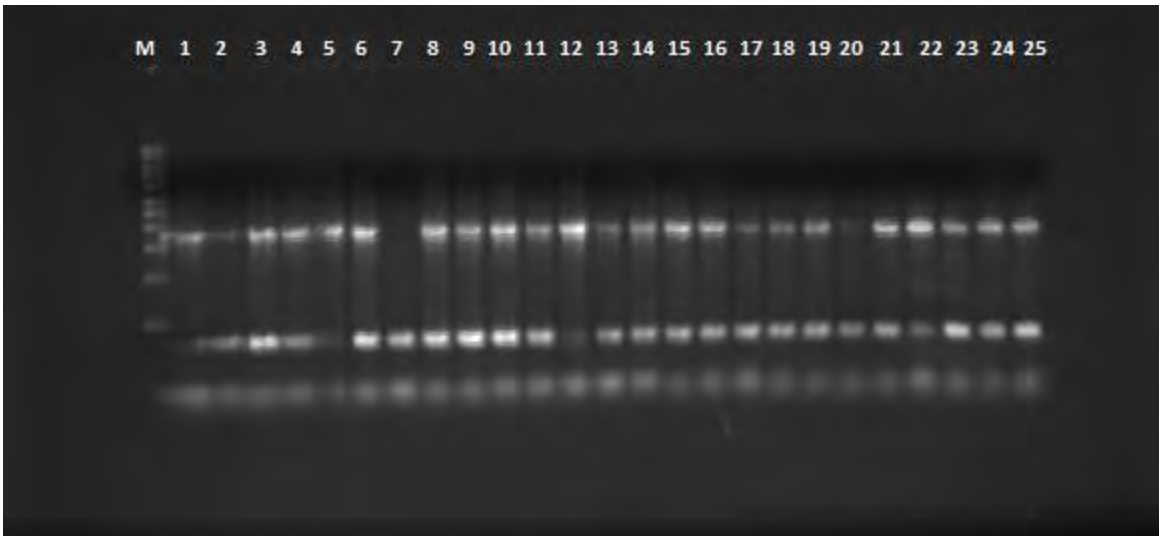
Yang, F., Andrew, J. G., Damon, C. H., Frank, J. W., Christi, A. W. and Jacqueline, J. C. (2000). Pulmonary expression of the human haptoglobin gene. *Am. J. Respir. Cell Mol. Biol.* **23**: 277–282.

Yang, Q., Liu, H., Low, H. Q., Wang, H., Yu, Y., Fu, X., Yu, G., Chen, M., Yan, X., Chen, S., Huang, W., Liu, J. and Zhang, F. (2012). Chromosome 2p14 is linked to susceptibility to leprosy. *PLoS One* **7**: e29747.

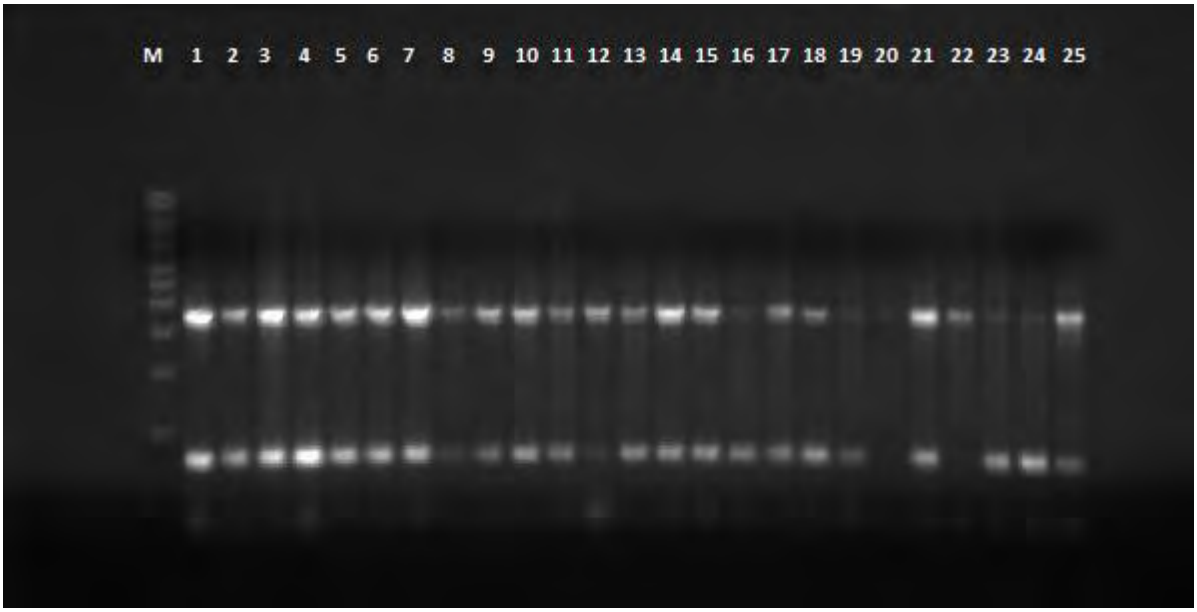
Zhu, J. and Paul, W. E. (2008). CD4 T cells: fates, functions, and faults. *Blood* **112**: 1557-1569.

Appendices

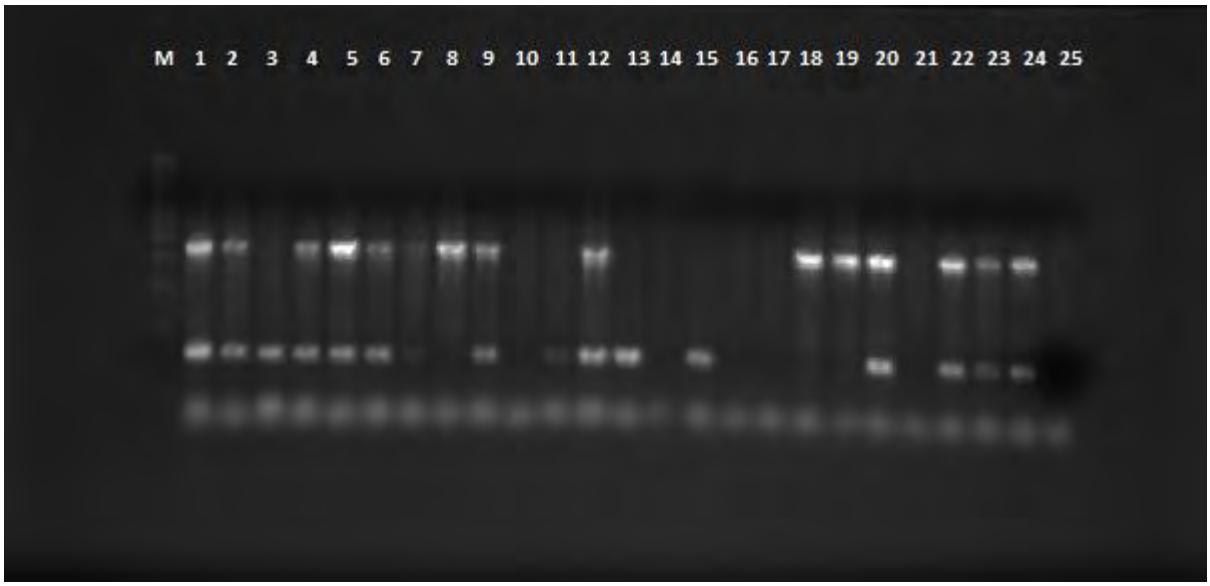
Appendix 1: PCR amplicons of Hp gene



M = size marker, 1 = ca-016, 2 = ca-017, 3 = ca-018, 4 = ca-019, 5 = ca-020, 6 = ca-023, 7 = ca-024, 8 = ca-026, 9 = ca-027, 10 = ca-028, 11 = ca-031, 12 = ca-032, 13 = co-064, 14 = co-086, 15 = co-073, 16 = co-061, 17 = co-071, 18 = co-083, 19 = co-022, 20 = co-012 , 21 = co-004, 22 = co-032, 23 = co-041, 24 = co-053, 25 = co-052



M= size marker, 1 = co-011, 2 = co-030, 3 = co-050, 4 = co-059, 5 = co-082, 6 = co-090, 7 = co-027, 8 = co-079, 9 = co-057, 10 = co-048, 11 = co-038, 12 = co-009, 13 = co-019, 14 = co-080, 15 = co-010, 16 = co-020, 17 = co-029, 18 = co-039, 19 = co-049, 20 = co-062 , 21 = co-054, 22 = co-045, 23 = co-087, 24 = ca-013, 25 = ca-014



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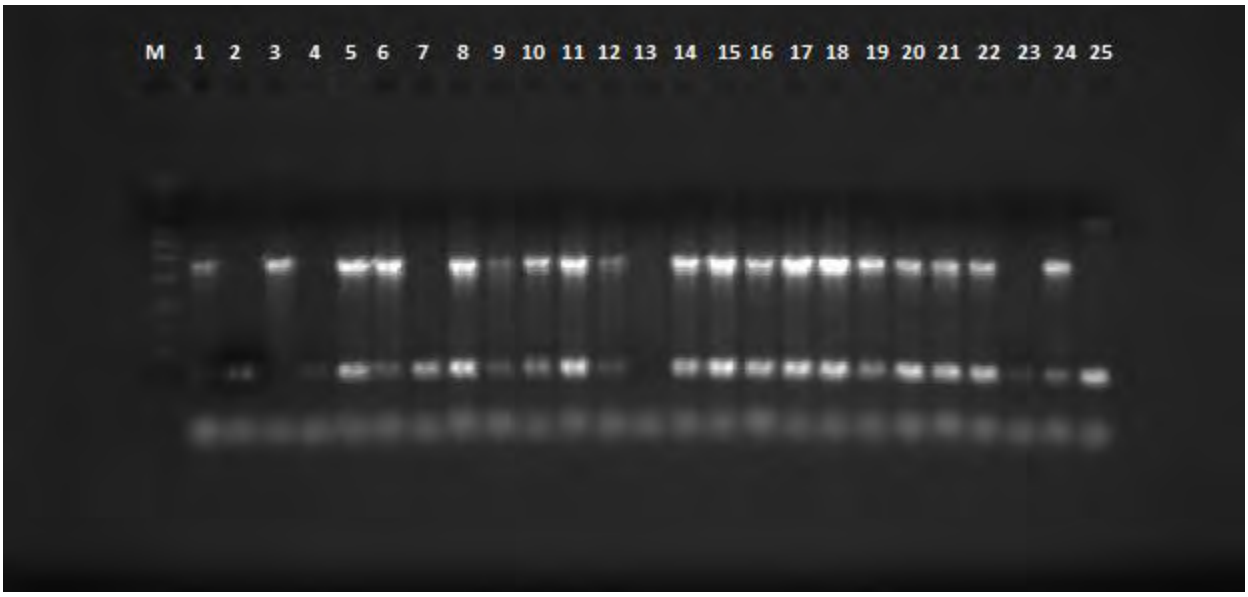
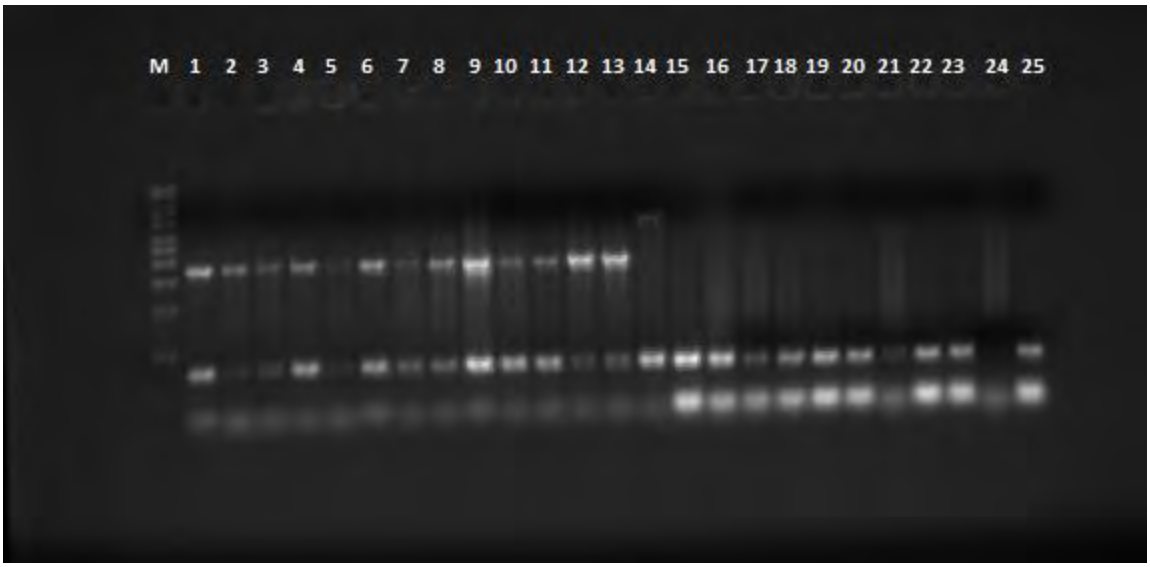


M = size marker, 1 = ca-033, 2 = ca-034, 3 = ca-035, 4 = ca-036, 5 = ca-037, 6 = ca-038, 7 = ca-039, 8 = ca-040, 9 = ca-041, 10 = ca-042, 11 = ca-044, 12 = ca-045, 13 = ca-046, 14 = ca-048, 15 = ca-049, 16 = ca-050, 17 = ca-053, 18 = ca-055, 19 = ca-057, 20 = ca-059, 21 = ca-060, 22 = ca-061, 23 = ca-062, 24 = ca-066, 25 = ca-067



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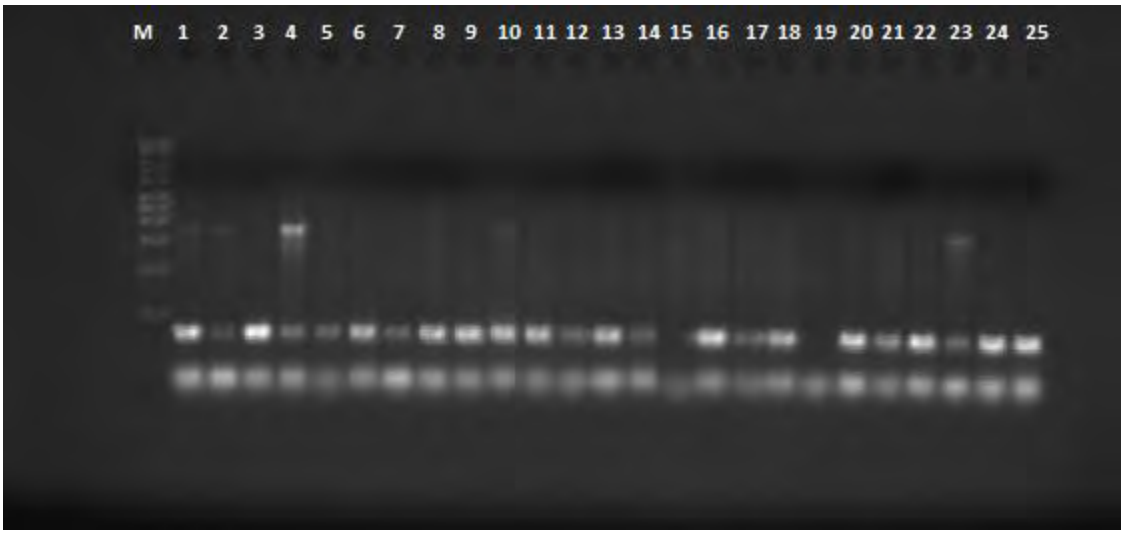
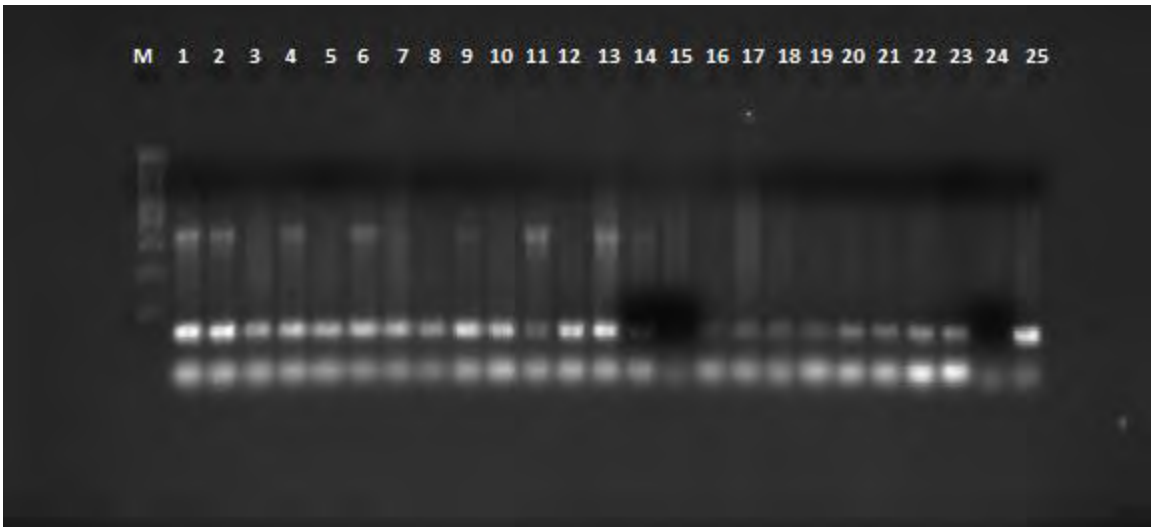


M = size marker, 1 = ca-009, 2 = co-006, 3 = co-015, 4 = co-023, 5 = co-033, 6 = co-036, 7 = co-037, 8 = co-040, 9 = co-044, 10 = co-047, 11 = co-056, 12 = co-065, 13 = co-066, 14 = co-067, 15 = co-069, 16 = co-072, 17 = co-074, 18 = co-075, 19 = co-076, 20 = co-077 , 21 = co-081, 22 = co-084, 23 = co-088, 24 = co-089, 25 = co-091



M = size marker, 1 = co-092, 2 = co-093, 3 = co-097, 4 = co-098, 5 = co-099, 6 = co-100, 7 = co-101, 8 = co-102, 9 = co-103, 10 = co-104, 11 = co-105, 12 = co-106, 13 = co-107, 14 = co-108, 15 = X1, 16 = X2, 17 = X3, 18 = ca-099, 19 = ca-100, 20 = ca-102 , 21 = ca-103, 22 = ca-104, 23 = ca-054, 24 = ca-080,25 = xxxxxx

Appendix 2: phases of study protocol

Phase I: blood sample collection

- **Materials:**
  - EDTA tubes [5ml]
  - Needles [20 gauge]
  - Needle holder/adaptor
  - Latex glove
  - Tourniquet
  - Alcohol wipes
  - Cotton wool
- **Procedure:**
  1. Ensure sample tubes are labelled with the subject code and the study visit number.
  2. Collect blood (5ml) samples according to standard clinical procedures
  3. Invert the tube carefully 10 times to mix blood and anticoagulant. Do not shake.

Phase II: plasma and Buffy coat preparation

- **Materials:**
  - Blue top tubes [15ml]

- Disposable plastic pipette [1ml] (or cut off 1ml pipette tip with its pipette)
- Latex glove
- Centrifuge
- 1.5 eppendorf tubes or lavender lid [for plasma]

- **Procedure:**
  1. Maintain sample at room temperature (18-22 °C) throughout processing.
  2. Samples should undergo centrifugation immediately. This should be carried out for a minimum of 10 minutes at 2000xg at room temperature. Do not use brake to stop centrifuge.
  3. This will give three layers: (from top to bottom) plasma, leucocytes (Buffy coat), and erythrocytes.
  4. Carefully aspirate the supernatant (plasma) at room temperature and add in 1.5ml eppendorf tube. Take care not to disrupt the WBC layer or transfer any cells. Do not take all the plasma; collection of all plasma may cause contamination with the underlying Buffy coat and red blood cell layers.
  5. Ensure that the 1.5ml eppendorf tube is adequately labeled with the relevant information and store at –80 °C.
  6. Using a cut-off 1ml pipette tip [or disposable plastic pipette (1ml)], collect the buffy coat layer into a separate 15 ml blue top tube and mix by pipetting up and down a number of times. The resulting sample will be enriched for white blood cells, but will also contain some of the overlying plasma and underlying red blood cells.
  7. Ensure that the 15ml blue top tube is adequately labeled with the relevant information and store at -80 °C.
  8. Save the RBC in EDTA for serological examination

Phase III: serological examination by slide technique

- **Materials:**
  - RBCs
  - Slides
  - Applicator sticks
  - Reagents
    - Anti-A
    - Anti-B

- **Procedure:**
  1. Prepare a suspension of test red blood cells
  2. Place on a labeled glass slide: 1 volume of Anti-A or Anti-B, or Anti-D reagent and 1 volume of test red cell suspension.
  3. Using a clear applicator stick, mix reagent and cells over an area of about 20 x 40ml.
  4. Slowly tilt the slide back and forth for 30 seconds, with occasional further mixing during the 2 minutes period, maintain the slide at room temperature.

5. Read macroscopically after 2 minutes over a diffuse light and do not mistake fibrin strands as agglutination.
6. Any test giving weak reactions should be repeated by the tube test technique.

Phase IV: DNA extraction

- **Materials, chemicals and reagents:**
  - Polypropylene tubes 15ml
  - Lysis buffer (10mM Tris-HCL,400mM NaCl, 2mM Na_2EDTA, pH 8.2)
  - SDS 10%
  - Proteinase K solution (1 mg proteinase K in 1% SDS and 2 mM Na_2 EDTA).
  - Centrifuge
  - Absolute ethanol
  - TE buffer (10mM Tris-HCL, 0.2mM Na_2 EDTA, pH 7.5)
  - Disposable gloves
  - Gilson pipette
- **Procedure:**
  1. Re-suspend the Buffy coats of nucleated cells obtained from blood with anticoagulents (ACD or EDTA) with 3ml of nuclear lysis buffer.
  2. Digest the cell lysates, with 0.2 ml of 10% SDS and 0.5 ml of proteinase K solution, overnight at 37 °C
  3. Add 1ml of saturated NaCl (6M) to each tube and shake vigorously for 15 seconds.
  4. Centrifuge for 15 minutes at 2500 rpm.
  5. Transfer the supernatant containing the DNA to another 15ml polypropylene tube, the precipitated protein pellet is left behind at the bottom of the tube.
  6. Add 2 volumes of absolute ethanol and invert the tubes several times until the DNA precipitates.
  7. Remove the precipitated DNA with a plastic spatula or pipette and transfer to a 1.5ml microcentrifuge tube containing 100-200 microliter TE buffer
  8. Dissolve the DNA for 2 hours at 37°C
  9. Store the tube at +4 or –20°C.
  10. Check quantity/quality of DNA (see QUALITY CONTROL OF DNA protocol)

Phase V: PCR reaction

- **Materials, chemicals, and reagents:**
  - HotStarTaq master mix kit
  - dsDNA templates
  - primers (forward and reverse)
  - mineral oil (if the thermo cycler does not have a heated lid)
  - PCR tubes
  - Pipettes
  - Pipette tips
  - Thermo cycler

- Vortex

- **Important points before starting**
  - HotStarTaq DNA Polymerase requires an activation step of 15 min at 95°C (see step6 of this protocol).
  - HotStarTaq Master Mix provides a final concentration of 1.5mM MgCl2 in the final reaction mix, which will produce satisfactory results in most cases. However, if a higher Mg2+ concentration is required, prepare a stock solution containing25mM MgCl2.
  - Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
  - Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- **Procedure**
  1. Thaw primer solutions:
     Mix well before use.
     Optional: prepare a primer mix of an appropriate concentration (see Table 1) using the water provided. This is recommended if several amplification reactions using the same primer pair are to be performed. The volume of primer mix added to each 50µl reaction is 25µl minus the volume of template DNA.
  2. Mix the HotStarTaq Master Mix by vortexing briefly and dispense 25µl into each **PCR** tube according to Table 1.
     It is important to mix the HotStarTaq Master Mix before use in order to avoid localized concentrations of salt. HotStarTaq Master Mix is provided as a 2x concentrate (i.e., a 25µl volume of the HotStarTaq Master Mix is required for amplification reactions with a final volume of 50µl). For volumes smaller than 50µl, the 1:1 ratio of HotStarTaq Master Mix to diluted primer mix and template should be maintained as defined in Table 1. A negative control (without template DNA) should always be included.
     It is not necessary to keep reaction vessels on ice since HotStarTaq DNA Polymerase is inactive at room temperature.
  3. Distribute the appropriate volume of diluted primer mix into the PCR tubes containing the Master Mix.
  4. Add template DNA (<1µg/50µl reaction) to the individual PCR tubes.
  5. When using a thermal cycler with a heated lid, **do not use mineral oil**. Proceed directly to step 6. Otherwise, overlay with approximately 100µl mineral oil.
  6. Program the thermal cycler according to the manufacturer's instructions.
     Note: Each PCR program must start with an initial heat activation step at 95°C for
     15 min.

A typical PCR cycling program is outlined in Table 2. For maximum yield and specificity, temperatures and cycling times should be optimized for each new template target or primer pair.

7. Place the PCR tubes in the thermal cycler and start the cycling program.
   Note: After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

Table1. Reaction composition using HotStarTaq Master Mix

Components			Volume/reaction	Final concentration
Hotstar taq master mix			10µl	2.5 units HotStarTaq DNAP
				1xPCR Buffer
				200µM of each dNTP
Primer A	Or	Primer C	1µl	0.5µM
Primer B		Primer D	1µl	0.5µM
RNase-free water			7µl	-
Template DNA			1µl	≈ 1µg/20µl reaction
Total			20µl	-

Table2. Optimized Cycling Protocol

95°C	15minutes	1 cycle
95°C	30seconds	15 cycles
69°C to 54°C	45seconds	
72°C	1minute	
95°C	30 seconds	35 cycles
55°C	45 seconds	
72°C	1 minute & 30se	
72°C	7minutes	1 cycle
4°C	Forever	(pick up samples)

Phase VI: gel electrophoresis

- **Materials, chemicals, and reagents**
  - Agarose
  - TAE Buffer
  - 6X Sample Loading Buffer
  - DNA ladder standard
  - Electrophoresis chamber
  - Power supply
  - Gel casting tray and combs
  - DNA stain
  - Staining tray

- Gloves
- Pipette and tips

Recipes: **TAE Buffer**

4.84 g Tris Base
1.14 ml Glacial Acetic Acid
2 ml 0.5M EDTA (pH 8.0)
- bring the total volume up to 1L with water

Add Tris base to ~900 ml H_2O. Add acetic acid and EDTA to solution and mix. Pour mixture into 1 L graduated cylinder and add H_2O to a total volume of 1 L.

Note – for convenience a concentrated stock of TAE buffer (either 10X or 50X) is often made ahead of time and diluted with water to 1X concentration prior to use.

6X Sample Loading Buffer
1 ml sterile H_2O
1 ml Glycerol
enough bromophenol blue to make the buffer deep blue (~ 0.05 mg)
-for long term storage, keep sample loading buffer frozen.

Quikview DNA stain
25 ml WARDS QUIKView DNA Stain
475 warm water (50-55° C)

A. Preparing agarose gel :

1. Measure 1.25 g Agarose powder and add it to a 500 ml flask
2. Add 125 ml TAE Buffer to the flask. (the total gel volume well vary depending on the size of the casting tray)
3. Melt the agarose in a microwave or hot water bath until the solution becomes clear. (If using a microwave, heat the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the flask).
4. Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.
5. Seal the ends of the casting tray with two layers of tape.
6. Place the combs in the gel casting tray.
7. Pour the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
8. Carefully pull out the combs and remove the tape.
9. Place the gel in the electrophoresis chamber.
10. Add enough TAE Buffer so that there is about 2-3 mm of buffer over the gel.

Note – gels can be made several days prior to use and sealed in plastic wrap (without combs). If the gel becomes excessively dry, allow it to rehydrate in the buffer within the gel box for a few minutes prior to loading samples.

B. Loading the gel:

1. Add 6 µl of 6X Sample Loading Buffer to each 25 µl PCR reaction
2. Record the order each sample will be loaded on the gel, including who prepared the sample, the DNA template - what organism the DNA came from, controls and ladder.
3. Carefully pipette 20 µl of each sample/Sample Loading Buffer mixture into separate wells in the gel.
4. Pipette 10 µl of the DNA ladder standard into at least one well of each row on the gel. Note – if you are running multiple gels, avoid later confusion by loading the DNA ladder in different lanes on each gel.

C. Running the gel:

1. Place the lid on the gel box, connecting the electrodes.
2. Connect the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected. (Remember – "Run to Red")
3. Turn on the power supply to about 100 volts. Maximum allowed voltage will vary depending on the size of the electrophoresis chamber – **it should not exceed 5 volts/ cm between electrodes!**
4. Check to make sure the current is running through the buffer by looking for bubbles forming on each electrode.
5. Check to make sure that the current is running in the correct direction by observing the movement of the blue loading dye – this will take a couple of minutes (it will run in the same direction as the DNA).
6. Let the power run until the blue dye approaches the end of the gel.
7. Turn off the power.
8. Disconnect the wires from the power supply.
9. Remove the lid of the electrophoresis chamber.
10. Using gloves, carefully remove the tray and gel.

D. Gel staining:

1. Using gloves remove the gel from the casting tray and place into the staining dish.
2. Add warmed (50-55°) staining mix.
3. Allow gel to stain for at least 25-30 minutes (the entire gel will become dark blue).
4. Pour off the stain (the stain can be saved for future use).
5. Rinse the gel and staining tray with water to remove residual stain.
6. Fill the tray with warm tap water (50-55°). Change the water several times as it turns blue. Gradually the gel will become lighter, leaving only dark blue DNA bands. Destain completely overnight for best results.
7. View the gel against a white light box or bright surface.
8. Record the data while the gel is fresh, very light bands may be difficult to see with time.

   Note – Gels stained with blue stains are stable for long periods. When destaining is complete, remove gel from water and allow the gel to dehydrate. Dark bands can be seen for in a dried gel for weeks or months.

Phase VII: quantification of plasma Hp level (ELISA)

- **Materials, chemicals, and reagents:**
  - Human haptoglobin ELISA test kit
  - Microplate reader capable of measuring absorbance at 450 nm.
  - Precision pipettes to deliver 1µl to 1 ml volumes and multiple channel
  - Distilled or deionized reagent grade water.
- **Preparation of reagents**

1. Freshly dilute all reagents and bring all reagents to room temperature before use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.
2. Diluent (10xs): Dilute the Diluent 1:10 with reagent grade water. Store for up to 1 month at 2 to 8°C.
3. Standard Curve: Reconstitute the Haptoglobulin Standard with the appropriate amount of Diluent to generate a solution of 40µg/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the standard solution (20µg/ml) 1:2 with Diluent, then 1:4 to produce 20, 5, 1.25, 0.313, and 0.078µg/ml solutions. Diluent serves as the zero standard (0µg/ml)Diluent serves as the zero standard (0µg/ml). Any remaining solution should be frozen at -20°C.

Standard points	Dilution	[Haptoglobin] (µg/ml)
P1	1 Part Standard (20µg/ml)	20.000
P2	1 part P1 + 3 part Diluent	5.000
P3	1 part P2 +3 part Diluent	1.250
P4	1 part P3 +3 part Diluent	0.313
P5	1 part P4 +3 part Diluent	0.078
P6	Diluent	0.000

4. Biotinylated Haptoglobulin (2X): Dilute Biotinylated Haptoglobulin with 4ml Diluent to produce a 2-fold stock solution. Allow to sit for 10 minutes with gentle agitation prior to making dilutions. The stock solution should be further diluted 1:2 with the Diluent. Any remaining solution should be frozen at -20^0C and used within 30 days.
5. Wash Buffer Concentrate (20X): Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
6. SP Conjugate (100X): Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with Diluent. Any remaining solution should be frozen at -20°C.

- **Assay method:**

1. Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30°C).

2. Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum dessicator.
3. Add 25µl of standard or sample per well, and immediately add 25µl of Biotinylated Haptoglobin to each well (on top of the Standard or sample) and mix gently. Cover wells with a sealing tape and incubate for one hour. Start the timer after the last sample addition.
4. Wash five times with 200µl of Wash Buffer manually. Invert the plate each time and decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid.
5. Add 50µl of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
6. Wash microplate as described above.
7. Add 50µl of Chromogen Substrate per well and incubate for about 10 minutes or till the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
8. Add 50µl of Stop Solution to each well. The color will change from blue to yellow.
9. Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Appendix 4: Some lab activities pictures