Haptoglobin polymorphism among *S. aureus* nasal carriage Birzeit University students.

By

Munther Abdallah

Supervisors

Prof. Dr. Joris Delanghe

Dr. Mohammad Farraj

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تعدد اشكال الهابتوجلوبين بين طلبة جامعة بيرزيت الحاملين لبكتيريا المكورات العنقودية المذهبة في الانف

By

Munther H. Abdalla

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Committee Members

Joris Delanghe PhD, Supervisor

Mohammed A. Farraj , PhD, Supervisor

Dr. Tamer Essawi, PhD. Internal Examiner

Wail Hammoudeh, MD., External Examiner

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Dedication

To my parents for their patience and support

To my wife and lovely children Samah, Mohammed, Loulou, Kind And Sara

To my dear brothers Khaled and Ashraf and my sisters for their supports and encouragement

To my dear friends for their great help and support

mmatani

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MHA

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List of Abbreviations

AEC	3-Amino-9-ethylcarbazole
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
apol1	Apolipoprotein 1
CBC	Complete blood count
CD163	Cluster of differentiation 163
CML	Chronic myelogenous leukemia
Dm	Diabetes Mellitus
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
Fe	Ferrum Iron
Fur	Ferric uptake regulator
H2O	Water
Hb	Hemoglobin
HCl	Hydrochloric acid
HO	Heme-oxygenase
Hp	Haptoglobin
Hp1F1S	Haptoglobin 1fast 1 slow
Hp 2FS	Haptoglobin 2 fast slow
Hp1S	Haptoglobin 1 slow

Hpr	Haptoglobin related gene
Hpx	Hemopexin
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IL-6	Interleukin-6
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
LDL	Low-density lipoprotein
M	Molar
mA	milliampere
ml	milliliter
Mn (II)	manganese
MRSA	Methicillin resistant *S. aureus*
NaOH	Sodium hydroxide
NO	Nitric oxide
O2	Oxygen
PCR	Polymerase Chain Reaction
PerR	Protein regulator
RBCs	Red blood cells
Rh	Rhesus factor

Rpms	Round per minute
S.aureus	*Staphylococcus aureus*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Th	T helper
TLF	Trypanosome lytic factor
TNF-α	Tumor necrosis factor alpha

Abstract

Staphylococcus aureus is a commensal as well as pathogenic organism. The carriage rate of *S. aureus* has been estimated to be 20-30% among Palestinian population. Nasal colonization of this organism can potentially increase the risk of infections. In bacteria, Fe is a co-factor of many enzymes and has crucial role in man physiological processes such as DNA replication, transcription and metabolism. Iron acquisition is required for *S. aureus* colonization and subsequent pathogenesis. *S. aureus* employ different mechanisms to obtain iron through lysis of the red blood cells by hemolytic toxins and the production of siderophores and the consumption of heme from the host.

Haptoglobin is an acute phase plasma protein that is synthesized primarily in the liver. There are two alleles expressing three different phenotypes, Hp1-1, Hp2-1 and Hp2-2. The polymorphism of haptoglobin has been associated with diseases in general and bacterial as well as other infections in particular. The main function of haptoglobin is seen in its rapid binding and removal of hemoglobin from the circulation. The presence of free hemoglobin in the circulation released due to hemolysis of the red blood cells in-vivo or during storage prior to transfusion has been associated with vascular injury.

This study was conducted on 1500 Birzeit university students, 26% males and 74% females. Nasal swabs whole blood were simultaneously obtained from all participants. Nasal swabs were cultured on mannitol salt agar to screen for the presence of *S. aureus*. Definitive identification of *S. aureus* was primarily done by tube coagulase and occasionally by Staphylase tests. Haptoglobin phenotyping was carried out by starch gel electrophoresis.

Our results showed that 20% of tested students were carriers for S. aureus. Haptoglobin polymorphism in S. aureus carriers was 12% Hp1-1, 34% Hp2-1 and 34% for Hp2-2. Haptoglobin polymorphism in control group was 10% Hp1-1, 44% Hp2-1 and 35% Hp2-2.

Haptoglobin polymorphism was not detected in 20% of cases and 10% of the controls. Statistical analysis using Chi square by SPSS did not reveal any significant difference between the cases and controls. Allele frequency calculated for haptoglobin was 0.37 for the Hp1 allele in both cases and controls.

The aims of this project were to determine the association of s. aureus nasal carriage with haptoglobin polymorphism. In addition, the allele frequency of haptoglobin gene was determined for the Palestinian study population.

In conclusion, this study has provided new information regarding the status of haptoglobin among young Palestinians who carried *S. aureus* in their anterior nares as compared to non carriers. We recommend that this study should be expanded using equal numbers of males and females. In addition, it should also consider involving patients suffering from diseases such as renal failure, cardiovascular disease and diabetes.

ملخص

تعتبر المكورات العنقودية الذهبية من البكتيريا المتعايشة علي جسم الانسان، وكذلك لها القدره على التسبب بعديد من الامراض. ويقدر معدل الناس الحاملين لهذه الجرثومه في فلسطين من 20-30٪. مع ارتفاع نسبة الاستعمار الأنفي بهذه البكتيريا من ازدياد خطر العدوى في العديد من الامراض.

يعتبر عنصر الحديد عامل مساعد للعديد من الإنزيمات وله دور حاسم في العمليات الفيسيولوجية مثل تكرار الحمض النوويDNA، والتمثيل الغذائي. الحصول عل الحديد يعتبر عامل اساسي لنمو وتكاثر البكتيريا بشكل عام والمكورات العنقوديه بشكل خاص . تستخدم والمكورات العنقوديه آليات مختلفة للحصول على الحديد من خلال تحلل كرات الدم الحمراء وتستخدم اليات مختلفه للحصول على الحديد منها صنع مجسات مستلمه للحديد وتسمى (siderophores).

الهابتوغلوبين هو بروتين البلازما الذي يتم تصنيعه في الكبد بشكل اساسي . هناك نوعان من الأليلات المسؤوله عن انتاج هذا البروتين وينتج عن هذة الاليلات ثلاثة اشكال مختلفة تدعى: Hp1-1، Hp2-1 وHp2-2.

ارتبط تعدد اشكال هابتوغلوبين بالعديد من الأمراض بشكل عام والالتهابات البكتيرية وكذلك الأمراض المعدية الأخرى بشكل خاص. وتعتبر الوظيفه الاساسيه للهابتوغلوبين هي ازاله والتخلص من الهيموجلوبين الحر في الدورة الدمويه .حيث يساعد في حماية الجسم من الكثير من الامراض التي يسببها الهيموجلوبين الحر في بلازما الدم.

أجريت هذه الدراسة على 1500 طالب وطالبه في جامعة بيرزيت، 26٪ ذكور و 74٪ إناث. بعد ان تم اخذ مسحات الأنف وعينات الدم في نفس الوقت من جميع المشاركين في البحث. تم زراعة المسحات الأنفية على Mannitol salt Agar للكشف عن وجود بكتريا المكورة العنقودية. ,لقد تم التأكد من جميع البكتيريا النامية على Mannitol salt Agar بواسطة فحص انزيم التخثر coagulase بالاضافة الى Staphylase test في بعض الاحيان.

أظهرت نتائج البحث أن 21٪ من الطلاب حاملين لهذه الجرثومه في الانف ،تم تحليل جميع العينات 224 عينه لحاملين للجرثومه و 99 عيينة اختيرت عشوائيا من الطلبة الغير حامليين للبكتيريا العنقوديه لتحديد التعدد الشكلي للهابتوغلوبين وكانت النتائج كالاتي . 12٪ Hp1-1، 34٪ Hp2-1 وHp2-2%34. كان تعدد الأشكال الهابتوغلوبين بين الطلبه الغير حاملين للجرثومه هو 10٪ Hp1-1، 44٪ Hp2-1 و 35٪ Hp2-2.تم تحليل النتائج باستخدام برنامج التحليل الإحصائي SPSS

وقد اثبتت الدراسه انه لا يوجد علاقه قويه بين تراكم البكتيريا العنقوديه بالانف ونوع الهابتوغلوبين الموجود بالدم , ولا يوجد اي فرق بين الطلبه الحامليين للجرثومه او الغير حاملين لها. تم احتساب Hp alleles في المجتمع الفلسطيني وتم مقارنته مع المجتعات في الدول المجاوره وكان مساويا ل 37%.

لقد كان الهدف الاساسي لهذه الدراسة ايجاد علاقة بين الحاملين للمكورات العنقودية الذهبية وتعدد اشكال الهابتوجلوبين في الجسم. اضافة لذلك تم تحديد Hp alleles frequency.

وفي الختام، قدمت هذه الدراسة معلومات جديدة فيما يتعلق بوضع هابتوغلوبين بين الشبان الفلسطينيين الذين حملوا المكورات العنقوديه المذهبة في الأنف بالمقارنة مع غير الحاملين. نوصي بأن يتم توسيع هذه الدراسة باستخدام أعداد متساوية من الذكور والإناث. وبالإضافة إلى ذلك، ينبغي ربط التعدد الشكلي للهابتوجلوبين بالعديد من الامراض الاخري مثل مرض السكري وامراض الكلى وامراض القلب والاوعيه الدمويه .

Chapter 1

Introduction

1.1. *Staphylococcus aureus*

S. aureus is a Gram positive coccus that has grape-like cluster morphology under the microscope. It is a commensal organism that resides in the skin, mucus membranes and the nose of healthy people. It has carotenoid pigment which gives the organism its characteristic golden yellow colonies during growth on rich media. The carotenoid pigment is considered a virulence factor which protects *S. aureus* from oxidants produced during the immune response. On media containing 5% sheep blood, *S. aureus* produces characteristic beta-hemolysis. *S. aureus* is a facultative anaerobic microorganism which is catalase positive, oxidase negative, coagulase positive, DNase positive and has tolerance to high concentrations of salt up to 1.7 M (Kaibni, Farraj, Adwan, & Essawi, 2009).

S. aureus is a commensal as well as pathogenic organism. The carriage rate of *S. aureus* has been estimated to be 20-30% among Palestinian population. Nasal colonization of this organism can potentially increase the risk of infections. When transmitted to food from food handlers who are also carriers of *S. aureus*, it forms toxins in the food and causes food intoxication. *S. aureus* is a major cause of community and nosocomial infections, it is considered the second leading cause of nosocomial bacteremia (Klein, Smith, & Laxminarayan, 2007). Humans and animals are the natural reservoir for *S. aureus*. *S. aureus* causes serious infection in hospitals and the community as well. *Methicillin Resistant S. aureus* (MRSA) have high mortality rates.

Few studies have reported a rate of *S. aureus* nasal carriage among Palestinians living in different regions in Palestine of 25%. MRSA accounted for 9% of the *S. aureus* isolates(Adwan

et al., 2013; Kaibni et al. (2009)).This high rate of MRSA carriage must be a priority for our medical research communities, due to the urgent need for developing better prevention ,diagnoses and treatment for infections with this pathogen. Since MRSA was first identified in 1961, it was not until the mid-1980s when *S. aureus* developed resistance to methicillin through the *mecA* gene (located on a mobile genetic element). After that, *S. aureus* in general and MRSA in particular became a major concern for their rapid spread in hospitals causing hard to treat nosocomial infections and in the community as well causing community acquired infections. It has been reported the presence of different susceptibility profiles between these two types of infections. In addition, it was found that Panton-Valentine Leukocidin gene is more predominant among community acquired strains(Shrestha, Singh, Raj, Pokhrel, & Mohapatra, 2014).

S. aureus has been considered one of the most common pathogens involved in causing hospital acquired infections (Velazquez-Meza, Hernandez-Salgado, Contreras-Cordero, Perez-Cortes, & Villarreal-Trevino, 2013). The pathogenesis of *S. aureus* is mediated by the production of many virulence factors. Production of virulence factors is dependent on the nature of nutrients available in the growth medium. The type of nutrients present in the growth medium is usually sensed by *S. aureus* and this can influence gene expression and the synthesis of virulence factors (Gilbert et al., 2013).

1.2. *Staphylococcus aureus* iron acquisition mechanism

Iron (Fe) is one of the most abundant elements in the Earth's crust and the most abundant transition metal in the human body. Iron is a vital element for the survival, growth and proliferation of nearly all organisms, including pathogenic bacteria. In bacteria, Fe is a co-factor for many enzymes and has crucial role in man physiological processes such as DNA replication, transcription and metabolism (Hammer & Skaar, 2011). Iron acquisition is required for *S. aureus* colonization and subsequent pathogenesis. *S. aureus* employs different mechanisms to obtain iron through lyses of the red blood cells by hemolytic toxins and the production of siderophores and the consumption of heme from the host as shown in Figure1. Siderophores are iron chelating compounds produced by *S. aureus* that bind iron with high affinity. Siderophore acquisition systems have an important role in staphylococcal pathogenesis, or they may represent a method of acquiring iron in non-pathogenic settings, such as during commensal colonization of the nasopharynx or skin(Skaar & Schneewind, 2004).

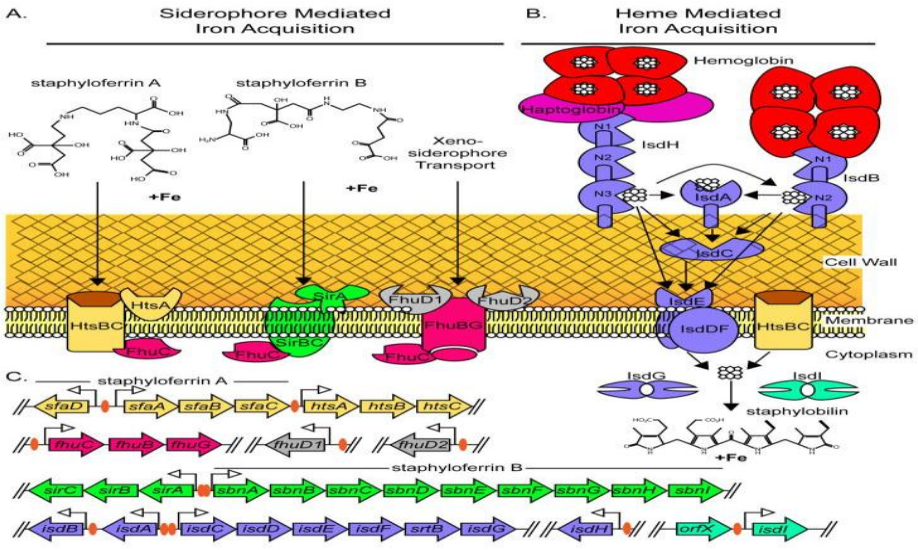

Figure 1: *Staphylococcus aureus* iron acquisition pathways(Hammer & Skaar, 2011)

1.3. Iron limitation and protection from bacterial infections

There is an ongoing battle for iron acquisition between our immune system and bacterial pathogens in general and *S. aureus* in particular. This has led to the development of mechanisms to limit iron availability to the invading microbes in a process termed as "Nutritional Immunity". Nutritional immunity is a term used to indicate withholding nutrients in order to inhibit bacterial growth. It is a form of defense mechanism adopted against microorganisms. The best example of nutritional immunity is intracellular sequestration of iron by ferritin, hemoglobin and myoglobin Figure 1. In addition, the free extracellular iron is usually insoluble and hence cannot be utilized by microorganisms(Hammer & Skaar, 2011). However, *Borrelia burgdorferi*, the causative agent of Lyme disease has evolved a defense mechanism against iron sequestering by substituting manganese for iron in its metal requiring enzymes(Posey & Gherardini, 2000). Other pathogens including *S. aureus* have evolved mechanisms to circumvent iron sequestering via expressing high affinity receptors (Hammer & Skaar, 2011; Skaar, Humayun, Bae, DeBord, & Schneewind, 2004).

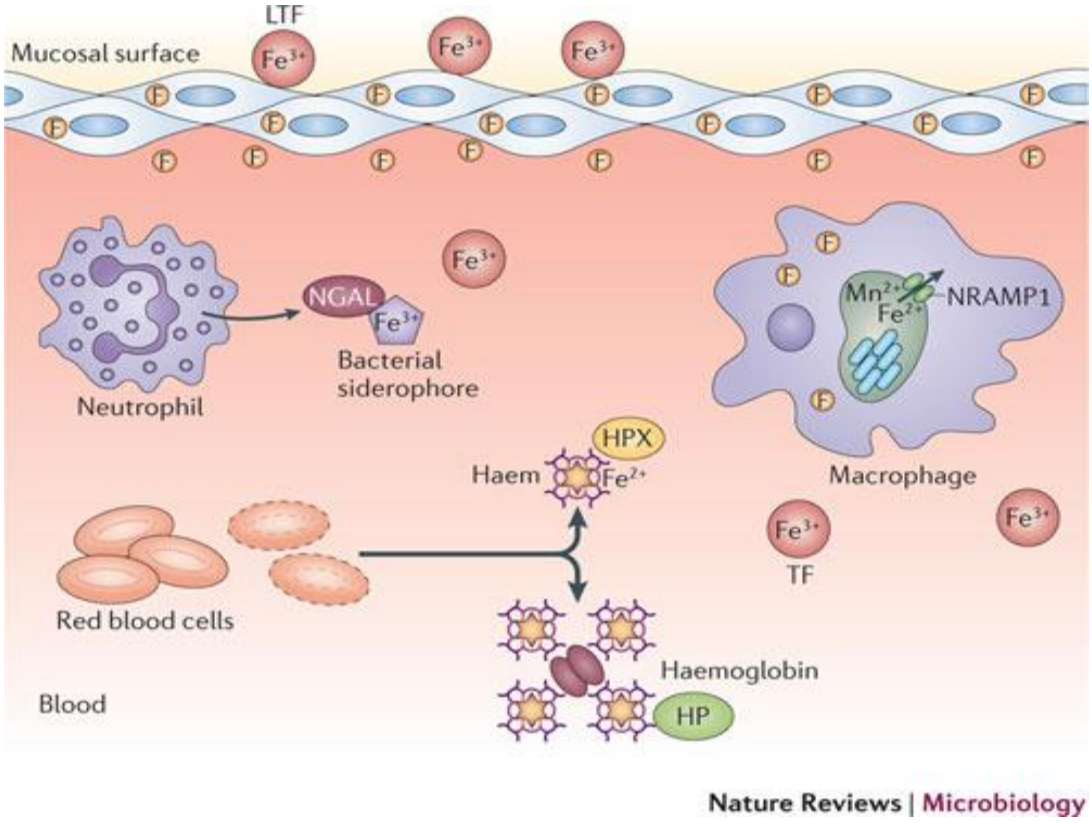

Figure 2: Fe limitation and Fe acquisition during bacterial infections (Hood & Skaar, 2012).

1.4. Metal-dependent regulation

Iron, magnesium, and manganese are metal ions that have a bacterial cofactor enzymatic function and in metabolism. These metals catalyze many biochemical reactions, maintain redox potential and produce harmful reactive oxygen species. Therefore, Bacteria have evolved mechanisms to maintain metal ion homeostasis. *S. aureus* regulates iron ion homeostasis by the ferric uptake regulator (Fur) and iron storage and antioxidant protein regulator (PerR) systems (Horsburgh, Clements, Crossley, Ingham, & Foster, 2001; Wu et al., 2006).

The main mechanism for *S. aureus* to obtain iron is siderophore-based iron acquisition which enables it to obtain iron from transferrin (Park, Sun, Choi, Bai, & Shin, 2005). It has been reported that defects in siderophore biosynthesis decrease the virulence of bacteria (Brozyna,

Sheldon, & Heinrichs, 2014). Regulation of siderophore biosynthesis and iron transport is mediated by ferric uptake regulator (Fur). Fur binds to or dissociates from a Fur promoter region on the DNA depending on the availability of iron (Horsburgh, Ingham, & Foster, 2001).When iron is present in a bacterium in high concentration; Fur binds to DNA and represses transcription of target genes. When iron concentration is very low, Fur dissociates in order to activate the target genes.

PerR, in *S. aureus,* is a transcriptional repressor of peroxide resistance operons (Horsburgh, Clements, et al., 2001). PerR is a redox sensor that can sense hydrogen peroxide using Mn (II) as a co-repressor (Traore et al., 2009). Based on the level of Mn(II) and iron, PerR has different functions. The PerR regulon is repressed in the presence of Mn(II), whereas the regulon is derepressed in the presence of iron (Morrissey, Cockayne, Brummell, & Williams, 2004).

1.5. Haptoglobin Synthesis and Structure

Haptoglobin (Hp) is an acute phase protein that is synthesized by the liver and found in the plasma. Hp synthesis is induced by cytokines produced during inflammation and in response to infections (Wang, Kinzie, Berger, Lim, & Baumann, 2001). Hp is composed of two chains, an alpha-chain (light) and beta-chain (heavy) that are linked by disulfide bonds between C131 on the alpha chain and C248 on the beta chain (Polticelli, Bocedi, Minervini, & Ascenzi, 2008). Three phenotypes of haptoglobin can be found in human plasma; Hp 1-1, Hp 2-1, Hp 2-2. As shown in Figure 3: Hp 2-1, Hp 2-2phenotypes exist as a series of polymers while the Hp 1-1 phenotype exists as a homogeneous protein. The haptoglobin types differ in their alpha or light chains. Hp 1-1 contains only α^1 chain, Hp 2-2 contains only α^2 chain and Hp 2-1 contains α^1 and α^2 chains.

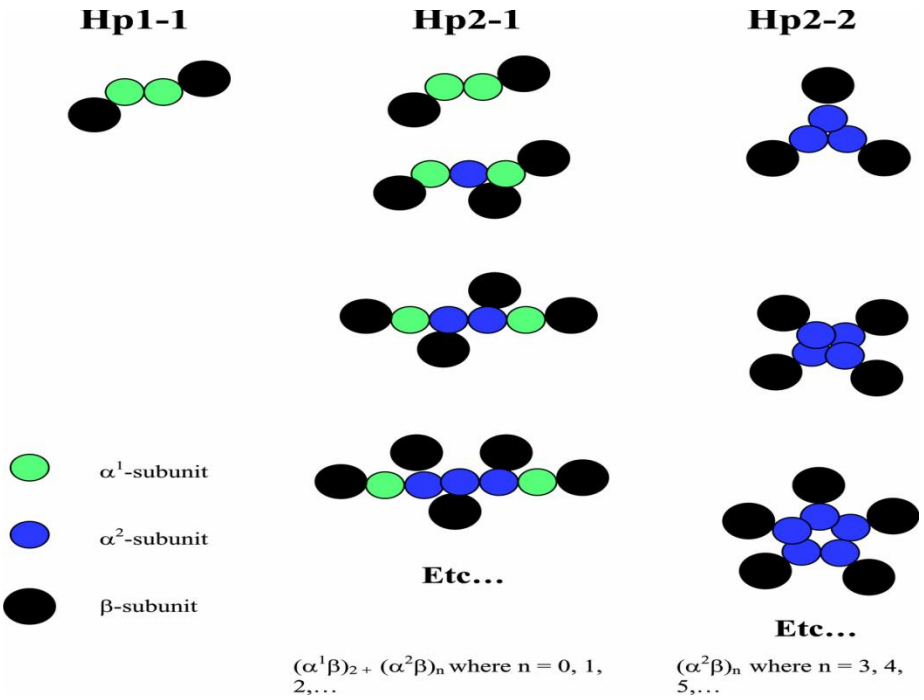

Figure 3: Subunit organizations of the major human Hp phenotypes

Haptoglobin usually increases one day after the original challenge and remains elevated for up to two weeks (Jacobsen, Andersen, Toelboell, & Heegaard, 2004).The haptoglobin response is proportional to the severity of the challenge (Jacobsen et al., 2004; Vels, Rontved, Bjerring, & Ingvartsen, 2009). The time course and extent of haptoglobin response is in concordance with the primary role of haptoglobin to prevent further tissue damage and promote tissue repair (Petersen, Nielsen, & Heegaard, 2004). Haptoglobin accomplishes its function by acting as antioxidant, anti-inflammatory agent, tissue-regeneration agent, bacteriostatic, and by regulating the maturation and activity of immune cells. Thus, haptoglobin has been proposed as indicator of acute and chronic diseases; however, the sensitivity of haptoglobin for disease detection is limited (Horadagoda et al., 1999). Reasons for the limited sensitivity of haptoglobin have been studied in cows. It was found that not all cows have elevated haptoglobin concentrations during

disease. Haptoglobin concentrations decrease after acute infection and it's response to tissue damage or infection is delayed (Horadagoda et al., 1999; Vels et al., 2009).The gene that encodes for haptoglobin is located on chromosome 16q22 (N. S. Levy et al., 2013; Polticelli et al., 2008). It consists of two main allelic forms; alpha-1 (1-1) that has 83 residues and alpha-2 (2-2) with 142 residues Figure 4. Polymorphisms in these two alleles have three possible genotypes; homozygous (Hp1-1 or Hp2-2) and heterozygous (Hp2-1). Three phenotypes of Hp have been identified; two alleles for Hp1 designated Hp 1F (fast) and HP 1S (slow) and one allele for Hp2 designated Hp 2FS. Hp1F and Hp1S alleles encode for exactly the same product except for one amino acid difference (Langlois & Delanghe, 1996). Hp2 allele evolved from crossing over between Hp1F and Hp1S alleles but it encodes a longer product. The Hp 2-2 phenotype has been associated with higher serum iron in healthy males only but not in females (Langlois, De Buyzere, Vlierberghe, & Delanghe, 2004; Langlois et al., 2000).

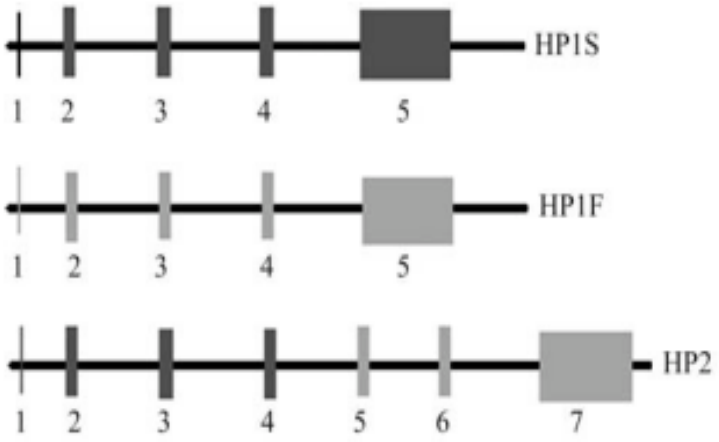

Figure 4: Haptoglobin gene structure (Yano, Yamamoto, Miyaishi, & Ishizu, 1998).

1.6. Other Haptoglobin structures

The existence of Hp structural variants have been recently described (Carter & Worwood, 2007). One variant, Hp-Carlberg, identified in 1958 was associated with reduced synthesis of the α1S chain (Quaye, 2008). Other variants such as modified Hp2-1 referred to as Hp2-1M differs from Hp2-1 by its electrophoretic mobility. The Hp2-1M phenotype has greater number of Hp1 bands thus makes it heavier and more intense (Connell & Smithies, 1959). This phenotype is generated by the polymorphism of a single nucleotide in the promoter region of the Hp2 gene and is more frequent in African populations (Maeda, 1991). Giblett (1959) reported that the prevalence of this phenotype in North American Blacks in the Seattle region was 9.8% (Giblett, 1961; Thomsen, Etzerodt, Svendsen, & Moestrup, 2013). Azevedo *et al.* (1969) studied a population of 541 Afro-descendants from northeastern Brazil and found an association between the $HP^{\alpha}2M$ allele and the presence of hypo-haptoglobinemia (Azevedo, Krieger, & Morton, 1969). The rare Hp Johnson is the result of a crossover between the two Hp2 alleles and causes hypohaptoglobinemia or ahaptoglobinemia. The electrophoretic pattern of this phenotype consists of the Hp1 band and various polymers that migrate slowly (Langlois & Delanghe, 1996; Smithies, Connell, & Dixon, 1962)

1.7. Haptoglobin-Related Gene (*Hpr*)

The Hp and haptoglobin related gene (*Hpr*) are located on chromosome 16 separated by 600 nucleotides Figur5. The *Hpr* gene sequence has evolved during evolution by gene duplication (Maeda, 1985). The *Hpr* gene sequence differs from that of the Hp gene by having inserted sequences from a retrovirus into the first intron. The entire *Hp gene* which includes the *Hpr* has 94% sequence identity (Hardwick et al., 2014). The active promoter region of the *Hpr* gene

encodes a haptoglobin-related protein (Hpr) (Maeda, 1985). The concentration of the Hpr in the human serum is about 5%-10% of that of Hp protein in healthy people.

The function of the Hpr protein is similar to that of the Hp protein in binding with high affinity the free hemoglobin found in the circulation. However, unlike Hp-Hb complexes, the Hpr-Hb complexes do not bind to the CD163 but remains in the circulation bound to apolipoprotein 1(apol1)(Nielsen et al., 2006). The Hpr plays an important role in protection in African population from *Trypanosoma bruceibrucei* infection due to its presence as part of the trypanosome lytic factor (TLF) (Maeda, 1985; Smith, Esko, & Hajduk, 1995).

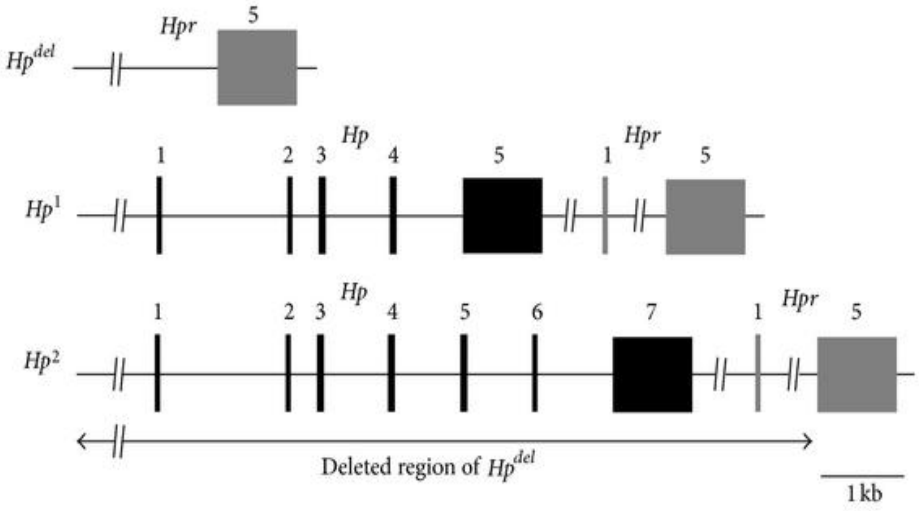

Figure 5: A schematic diagram of the genetic structure of the Hp^1,Hp^2 , and Hp^{del} alleles(Ko et al., 2013).

Excess of free serum Hb usually occurs subsequent to extensive intravascular hemolysis. This results in the depletion of the Hp levels while the Hpr levels don't differ significantly (Nielsen et al., 2006). The reason is the Hp-Hb complexes are internalized in the macrophages via the

CD163 receptor while the small amounts of Hpr-Hb complexes linger in the circulation bound on apol1**.**

1.8. Hp0 Phenotype

The Hp0 phenotype is characterized by the absence or reduced levels of Hp in plasma (Koda et al., 2000). This phenotype is genetically determined due to allelic deletion of the Hp gene in some individuals or low level of transcription in others resulting in conditions referred to as ahaptoglobinemia and hypohaptoglobinemia respectively (J. Delanghe, Langlois, & De Buyzere, 1998; Koda et al., 2000). Secondary hypohaptoglobinemia can be caused by non-genetic factors related to increased removal or reduced production of Hp during intravascular hemolysis and or liver diseases.

The nature of the genetically determined hypohaptoglobinemia in East Asian population results from a 28 kb deletion referred to as Hp^{Del} that stretches from promoter region of the HP gene to exon 5 of the HPR gene. This deletion is usually found in patients diagnosed with ahaptoglobinemia who developed anaphylactic transfusion reactions caused by antibodies against haptoglobin (Koda et al., 2000). The ahaptoglobinemia or complete absence of serum Hp corresponds with the homozygous genotype Hp^{Del}/Hp^{Del}. The hypohaptoglobinemia or extremely low serum levels of Hp correspond with $Hp2/Hp^{Del}$ genotype. The hypohaptoglobinemia or decreased levels (5o% less than normal) corresponds with$Hp1/Hp^{Del}$ genotype. The frequency of Hp^{Del} gene is found among the Japanese and South East Asians, are between 0.15 and 0.30 (Koda et al., 2000) and 0.8 among Mongolians (Soejima, Koda, Fujihara, & Takeshita, 2007). This genotype has not been identified in other Asian populations from Central, Southeast, South and West Asia. The lack of detection of the Hp^{Del} among these Asian populations can be due to

the sample size or the extinction of the Hp^{Del} allele. It has been reported that Hp^{Del} originated in China and then spread into Mongolia, Korea and Japan (Soejima et al., 2007).

The Hp^{Del} allele has not been found in European and African populations. Congenital ahaptoglobinemia among these populations can be caused by mutations in the promoter region of the HP gene(Teye et al., 2003). The prevalence of the Hp0 phenotype among Caucasians, is about 0.1% (Langlois & Delanghe, 1996), while as high as 40% in Africans (Teye et al., 2006); the occurrence of this phenotype is influenced by acquired ahaptoglobinemia in areas where malaria is endemic and untreated (Boreham et al., 1979). In North Americans of African descent, the frequency of the Hp0 phenotype is ~2.3% (Carter & Worwood, 2007).

1.9. Hemoglobin Structure and Function

The hemoglobin (Hb) molecule is found in red blood cells and consists of four polypeptide chains or globins. There are two alpha and two beta chains ($\alpha 2\beta 2$) each carry heme molecule containing ferrous iron (Fe^{++}). The structural and functional unit of adult hemoglobin is a tetramer of the 2 alpha and 2 beta chains binding together. Hb constitutes about one third of the mass of the red blood cell. The normal adult human hemoglobin is a mixture of 96-98% hemoglobin A ($\alpha 2\beta 2$), 1.5–3.2% HA2 ($\alpha 2\delta 2$) and 0.5-0.8% HbF ($\alpha 2\gamma 2$).

The normal function of Hb is the transport of oxygen from the lungs to tissues and removing carbon dioxide from tissues to the lungs to be expelled during expiration (Schechter, 2008). The quaternary structure of Hb dictates its ability to bind oxygen in the lungs and release it in the tissues. In the lungs, oxygen binds to the ferrous iron of the heme molecule and released in tissues requiring oxygen.

Hb genes are located on different chromosomes. The beta globin genes are located on chromosome 11 while the alpha globin genes are located on chromosome 16 as shown in Figure 6.

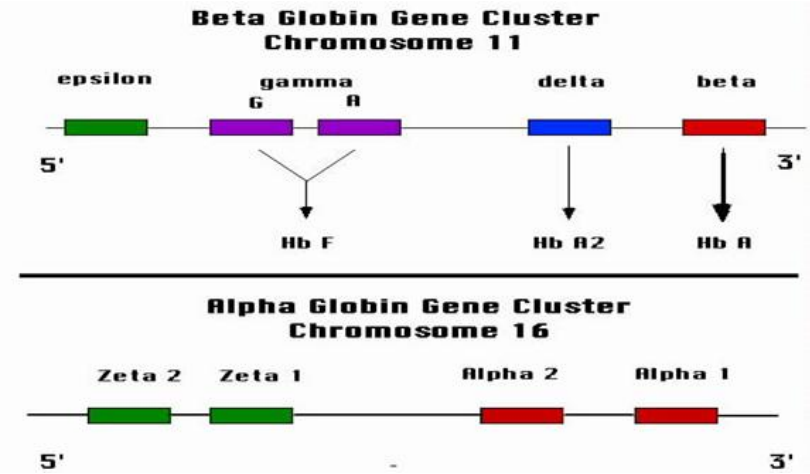

Figure 6: Beta globin gene cluster (Bunn, 1986)

1.10. Hemoglobin-Haptoglobin Binding

The binding of haptoglobin to hemoglobin is a significant event. Hemoglobin binding to haptoglobin was determined to occur on the alpha-chain of hemoglobin specifically on peptide 121-135 (Kazim & Atassi, 1981). Recent evidence indicates that Hp preferentially binds to the beta subunits of hemoglobin. Haptoglobin is α2-acidic serum glycoprotein which form complexes with hemoglobin with very high binding affinity (Kd = 10^{-12} M)(Polticelli et al., 2008). It is present in most body fluids in human and other mammals. The levels of Hp is usually increased during inflammation and malignancies but decreased during hemolytic conditions (Dobryszycka, 1997).

The main function of haptoglobin is seen in its rapid binding and removal of hemoglobin from the circulation. The presence of free hemoglobin in the circulation released due to hemolysis of the red blood cells in-vivo or during storage prior to transfusion has been associated with

vascular injury (Vermeulen Windsant et al., 2012). Hemoglobin ability to bind nitric oxide (NO) can inhibit NO signaling. In addition, haptoglobin binding to free hemoglobin inhibits its oxidation and thus prevents acute kidney injury (Schaer, Buehler, Alayash, Belcher, & Vercellotti, 2013). Therefore, haptoglobin can be considered as potential therapeutic agent for conditions involving acute and chronic hemolysis of erythrocytes (Schaer et al., 2013). The removal of Hp-Hb complex from the circulation is mediated through binding to the scavenger receptor CD163 Figure 7 carried on peripheral blood cells and tissue monocytes and macrophages (Buehler et al., 2009). The ability of haptoglobin to deprive microorganisms from iron needed to cause infections and its binding to free hemoglobin endows haptoglobin additional properties as an antibacterial agent and an antioxidant. Other heme binding proteins are recognized and well described in literature such as Hemopexin and albumin which function in sequestering free heme. Hemopexin-Hb complex is removed from circulation by neutrophiles and macrophages via CD91receptor as shown in Figure 7 below (Ascenzi et al., 2005; Baker, Anderson, & Baker, 2003; Thomsen et al., 2013).

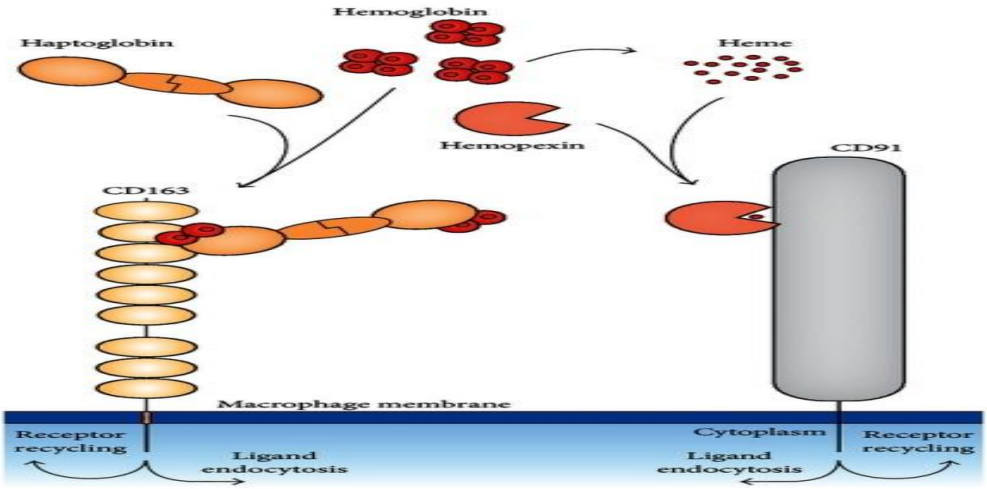

Figure 7: Pathway for Hb-Hp complexes and hemopexin- (Hx) heme complexes uptake. Adopted from (Thomsen et al., 2013)

1.11. Geographic Distribution of Haptoglobin Alleles

The frequency of Hp genes among different populations is geographically dependent (Giblett, 1961). The origin of the Hp2 allele for example has been originally reported in India and spread worldwide. This can be due to intense genetic pressure where the Hp2 allele became dominant and may have a selective advantage over the Hp1 allele (Schultze & Heremans, 1966). The frequency and the geographic distribution of the Hp1 allele are evident in many countries such as Southeast Asia, Europe, African and America. The highest frequencies of Hp1 allele have been found among indigenous populations residing in South America (Nagel & Etcheverry, 1963; Schultze & Heremans, 1966; Sutton, Matson, Robinson, & Koucky, 1960).

There is a state of constant equilibrium in the polymorphism of the Hp1/Hp2 throughout the world. Reports of allele frequencies of Hp1 and Hp2 vary between Europe and the American population Table 1. A frequency for Hp1 of 0.43 and0.54 and for Hp2 of 0.57 and 0.46 has been reported among European and American populations respectively (Langlois & Delanghe, 1996). The frequency of these two alleles had been studied among populations from southern and southeastern Brazil and revealed allele frequencies of 0.53 and 0.46 for Hp1 and 0.47 and 0.54 for Hp2 respectively (de Souza et al., 2003; Zaccariotto et al., 2006). Shreffler and Steinberg (1967) reported frequencies of 0.48 and 0.47 for Hp1 and 0.52 and 0.53 for Hp2 among Xavante Indians living in central-western Brazil (Shreffler & Steinberg, 1967). Among Indians of the Amazon region in South America, Simoes*et al.* (1989) reported high frequencies of the Hp1S allele and low frequencies or complete absence of the Hp1F allele (Simoes et al., 1989). Several studies conducted to determine the frequency of the Hp alleles among different populations worldwide is summarized in Table 1 and in Asian populations in Figure 8

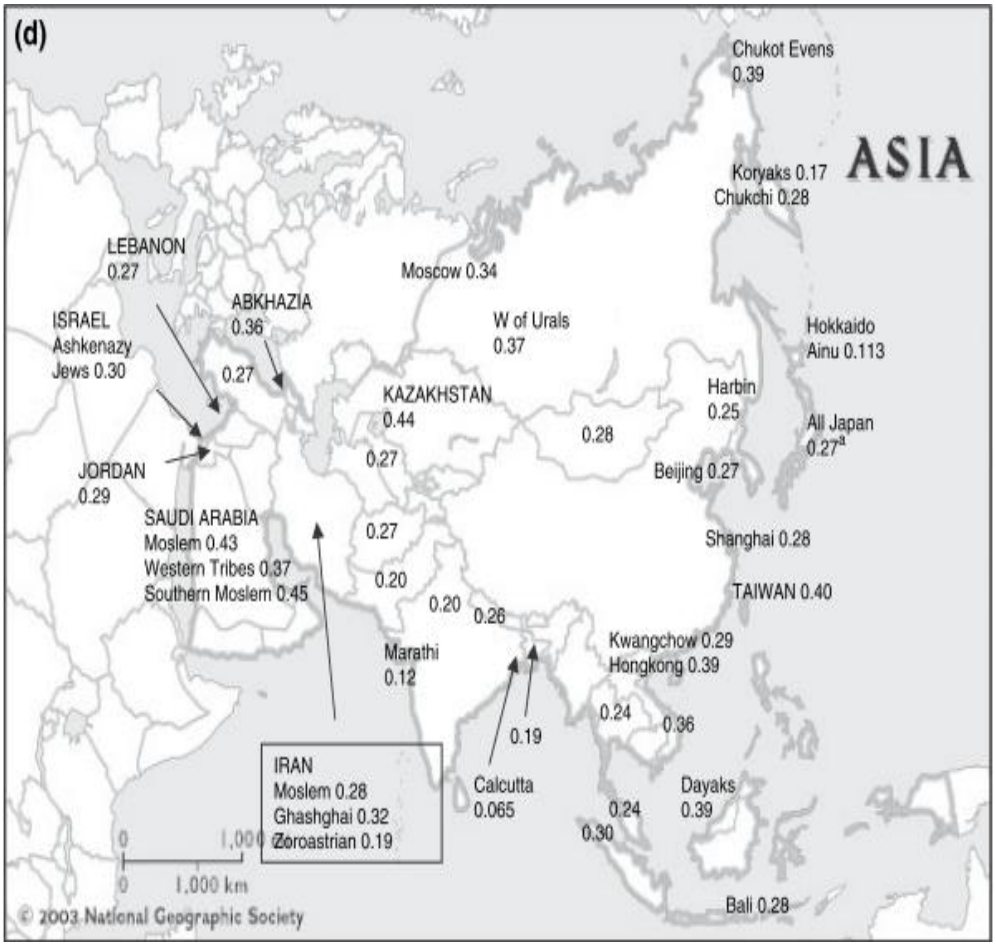

Figure 8: Hp1 Allele frequency among Asian population(Carter & Worwood, 2007)

Table 1: Hp1 gene frequencies in different populations .adopted from(Sonati, 2007).

Continent	Country	Population studied	No.	Hp1 allele frequency	Reference
Africa	Algeria (Sahara)	Saoura Valley region HoggarMontains region	373 260	0.64 0.66	Constans et . al . 1981
	Central African Republic	Pygmies Sahara region	919 280	0.35 0.53	
	Ghana	General population	123	0.52	Teye et .al . 2006
	Kenya	Luo tribe (north of Lake Victoria)	227	0.57	Herzog et .al. 1970
	Mali	Menaka region	285	0.44	Constans et.al.1981
	Nigeria	Ibadan town Zaria city	63 56	0.73 0.38	Shirn and Bearn.1964 Mastans et.al.1994
	Sudan	General population	208	0.54	Elagib et.al.1981
	Senegal	Bedik group Malinkes Peulhs	733 404 169	0.73 0.64 0.70	Constans et.al.1981
America	Brazil	Two populations (Costa da Lagoa and Sao Joao do Rio Vermelbo) from Santa Catarina Island. southern Brazil { descended from immigrants from the Azores Archipelago – ago(Portuguese) . with a minor contribution of sub – Saharan Africans and Amerindians	266	0.52 and 0.53 . respectively	Souza et.al.2003

	Canada	Caueasoids and Afro-descendants from southeastern Brazil Eskimos from beffinIsiand Mixed(French-canadians-africans-asians and others)	142 67 358	0.46 0.24 0.39	Zaccariotto et .al.2006 Shim and Bearn .1964 Mahmoud et.al.1994
	Chile	Araucanian Indians	31	0.77	Shim and Bearn.1964
	Colombia	Spanish-origin population from Bogota	109	0.43	Mastana et.al.1994
	USA	Indian (Alabama Coushatta) Eskimos(northem and southem Alaska) General population	143 220 3273	0.37 0.32 0.39	Shim and Bearn 1964 Scott et.al.1966 Levy et.al.2004
	Mexico	Durango State(Urban Indians) Durango State(Isolated Indians: (Tepehuanos.Huicholes.Tarahumara	119 206	0.63 0.62	Delanghe et.al.2000

		and Mexicancros) Maya Indian (Itza,Mam,Quiehe,Chol,Tzotzil,Tzeltal and Cakchiquel tribe) Maya (Lancandon tribe) Non-Maya Indians (Zapoteca.Chiapancea.Totonaca.Mestizo tribes)	 418 31 189	 From 0.50 to 0.69 0.93 From 0.53 to 0.67	 Sutton et.al.1960
	Peru	Cashinahua Indians	128	0.74	Johnston et.al.1969
	Venezuela	Piaroa Indians Makiritare Indians	121 186	0.82 0.49	Marini et.al.1993 Tanis et.al.973
Asia	Afghanistan	Tajik group Pushtoons Hazaras Usbeks	310 210 172 124	0.26 0.26 0.28 0.25	Rahimi et.al.1977
	China	Shenyang and Guangzou cities(northeastem and southemChina,respectively) General population Mongolian group	57 667 204	0.31 0.33 0.26	Teye et.al.2006 Ko et .al.1980 Zhao et.al. 1993
	India	Bengalee Hindus(hetcrogeneous caste	140	0.19	Bandopadhaya

		composition)			et.al.1992
		Punjab(northern India)-jat Sikh group	192	0.23	Kaur et .al.1981
		Punjab(northern India)-Khatri group	105	0.19	
		Punjab(northern India)- Balmiki group	108	0.14	Mastana et.al.1994
		Hindus from Hyderabad	116	0.20	
	Israel	General population	757	0.33	Lavie et .al.2003
	Japan	Mixed(two different Japanese population)	1211	0.26	Shindo . 1990
		General population	372	0.27	Nakada and Abe 1987
	Jordan	Jordanian Afro-descendants	163	0.36	(Janaydeh, Hamad, & Awadallah, 2004)Janaydeh et.al.2004
		General population	200	0.29	Awadallah and Hamad.2003)
	Korea	General population(male athletes)	120	0.31	Kang et.al.2003
		General population	316	0.32	Yang et.al.2000
	Malaysia	General population	231	0.30	Saha and Ong

					1984
	Russia	Chukotka Evens(northeastem Russia)	314	0.39	Solovenchuk and Glushenko 1985
	Thailand	North-central and northeastem areas(male members of the Royal Thai Army) General population (blood donors)	682 200	0.24 0.25	Blackwell and Thephusdin.1963 Shimada et.al.2007
	Turkey	General population	200	0.27	Erdem and Aksoy 1997
	Ukraine	Mixed (from Dnepropetrovsk .Kharkov.Odessa.Kiev.Uzhgorod.Zhitomir)	596	0.38	Nikolchcnko et.al. 1997
Europe	Belgium	General population	918	0.40	Van Vlierberghe et.al. 2001
	Germany	Hamburg (northern Germany)	1725	0.39	Kruger and Puschel 1993
	Greece	Northern Greece	212	0.35	Stromatias et.al.1987
	Hungary	Budapest General population	343 384	0.35 0.35	Hever 1976 Papp et.al.2007
	Italy	Continental Italy (north eastern,northwestern,central and southern regions) Sardinia	441 165	0.11 0.18	Santoro et.al.1983

	Moldavia	Gaugauz	190	0.35	Varsahr et.al.2001
	Norway	Oslo	6668	0.38	Teige et.al.1992
	Spain	Barcelona	317	0.38	Moral and Panadero 1983
	United Kingdom	Northeastern England	101	0.41	Mastana et.al.1994
		Southern Wales	265	0.40	Carter et.al.2003
Oceania	Australia	Caucasian population	307	0.40	Lai et.al.1976
		Aborigenes from Amhem Land	50	0.29	Shim and Bcarn.1964
		Aborienes from westem desert	101	0.19	
	Micronesia	Marshall Island	364	0.52	Neel et.al.1976
	Papua New Guinea	Southern Highland	99	0.80	Hill et.al.1986
		Eastern Highlands	59	0.53	
		Western Highlands	24	0.87	
		North Coast	22	0.64	
	Vanuatu	Espiritu Santo	79	0.73	
		Pentecost	101	0.68	
		Emae	60	0.63	
		Central group	70	0.61	
		Tanna	47	0.71	
		Futuna	32	0.69	
		Aneityum	46	0..61	

1.12. Haptoglobin Functions

Haptoglobin synthesis occurs mainly in the liver and in fewer amounts in the adipose tissue and lungs as well. The function of haptoglobin is seen in playing significant role as antioxidant and in antimicrobial activity. Haptoglobin plays a role in stimulation of angiogenesis and has highly potent cholesterol crystallization-promoting activity. Probably the most important biological function of haptoglobin has been manifested in the host defense responses to infection and inflammation, acting as a natural antagonist to receptor-ligand activation of the immune system.

The functions of the Hp phenotypes are related to the various biochemical and biophysical characteristics, as well as their functional efficiencies which determine their specific antioxidant and immunomodulatory capacities Table 2 (Langlois & Delanghe, 1996; H. K. Louagie, Brouwer, Delanghe, De Buyzere, & Leroux-Roels, 1996). The main biological functions of Hp are discussed in the following sections.

Table 2: Functional properties of Hp phenotypes(Langlois & Delanghe, 1996)

Function	Hp1-1	Hp2-1	Hp2-2
Hemoglobin binding	Strong	intermediate	Weak
Antioxidative capacity	Strong	intermediate	Weak
Inhibition of prostaglandin synthesis	Strong	intermediate	Weak
Angiogenic effect	Weak	intermediate	Strong
Agglutination of	-------	intermediate	Strong

Streptococcus pyogenes14			
Affinity towards CD22	Strong	Strong	Strong

1.12

.1. Haptoglobin-Hemoglobin binding and renal failure protection

Free hemoglobin released from RBCs highly toxic and mediates iron-driven oxidative stress and inflammation (Tseng, Lin, Huang, Liu, & Mao, 2004). It is known that the role of Hp is the removal of free Hb released during intravascular hemolysis. The Hp-Hb binding is irreversible, non-covalent and the complexes are soluble as characterized by high stability and affinity at 1 x 10^{-15}mol L^{-1}(Okazaki, Yanagisawa, & Nagai, 1997). Studies suggested that Hb-β chain has two Hp binding sites (residues β11-25 and b131-146) whereas the α-chain has only one Hp binding site (residues α121-127). The dimers of Hb αβ and Hpαβ subunits bind stoichiometrically (Langlois & Delanghe, 1996). Circulating haptoglobin reaches saturation at a free Hb concentration of 500-1500 mg L^{-1} (Van Vlierberghe, Langlois, & Delanghe, 2004).

The free Hb released from lysed RBC's is removed by glomerular filtration possibly resulting in renal damage. The role of Hp in binding Hb and iron is protective from renal damage because the Hp-Hb complex is not filtered through the glomeruli (Langlois & Delanghe, 1996; Van Vlierberghe et al., 2004). Most of the Hp-Hb complex (90%) is removed from the circulation by hepatocytes while 10% is removed by tissue monocytes/macrophages. Although the receptor for the Hp-Hb complex on hepatocytes has not been well characterized, but it has a high affinity for binding the Hb-Hp complex (Kino et al., 1980). The receptor for Hb-Hp has recently been identified in macrophages as CD163 (Horn et al., 2003; Kristiansen et al., 2001). The Hb-Hp

complex is usually enters the lysosomes via endocytosis and subsequently broken down. Although haptoglobin is not recycled, heme is degraded by heme-oxygenase (HO) to release iron, which is utilized in the synthesis of other proteins (Van Vlierberghe et al., 2004). Interleukin-6 has an important role in the regulation of this process because it stimulates Hp production and increases the expression of CD163 in macrophages increasing the efficiency of Hb-heme group degradation (Dennis, 2001).

1.12.2. Reduction of free toxic radicals

Free iron can react with oxygen (O2) via the Fenton reaction to generate superoxide, and with H_2O_2 to produce hydroxyl free radicals (Kaplan, 2002). The harmful effects of LDL are catalyzed by free iron thus causing vascular and endothelial cell damage (Grinshtein, Bamm, Tsemakhovich, & Shaklai, 2003). The damage to the vascular system by free radicals can be reduced by haptoglobin. This reduction is highly dependent on Hp phenotypes (Gutteridge, 1987; Van Vlierberghe et al., 2004). It has been reported that Hp1-1 provides greater protection against oxidative damage *in vitro* (Koda, Soejima, Yoshioka, & Kimura, 1998; Koda et al., 2000). The three Hp phenotypes have the same binding affinities for Hb (Melamed-Frank et al., 2001). Variations in size between the proteins play a role in their ability to prevent the release of heme. Since Hp2-2 phenotype is larger in size, it removes iron to the extravascular space more slowly. This causes greater oxidative stress in individuals who carries the Hp2-2 phenotype whose free Hb remains for longer time in the circulation (Melamed-Frank et al., 2001).

1.12.3. Nitric Oxide inhibition

Nitric oxide (NO) is a highly reactive gas produced by various cells such as endothelial cells and macrophages (Hibbs, Taintor, Vavrin, & Rachlin, 1988). NO is originally referred to as endothelium-derived relaxing factor (EDRF). The role that NO gas plays in the human body includes the maintenance of vascular tone and modulation of neurotransmitter function in the central and peripheral nervous systems, platelet aggregation and cellular defense (Green, 1995; Moncada & Higgs, 2006; Sadrzadeh & Bozorgmehr, 2004). Free Hb and Hp-Hb complex inactivate NO/EDRF, whereas Hp alone doesn't. Consequently, an increase in the level of circulating Hp-Hb may inhibit NO formation and endothelium relaxation, thereby enhancing the risk of cardiovascular disease (Moncada & Higgs, 2006). The Hp1-1 phenotype may be advantageous in this respect because the Hp1-1: Hb complex is removed from circulation more rapidly than the other Hp complexes (Melamed-Frank et al., 2001; Saeed, Ahmad, & Ahmed, 2007).

1.12.4. Hp phenotypes effects on immune system

As an acute phase protein, Hp production is usually enhanced during inflammation, infection or tumor growth. Hp participates actively in several processes of the immune response, from activation of the innate and adaptive immune responses to tissue repair and regeneration (Quaye, 2008).It has been well documented that Hp has immunoregulatory properties, with Hp2-2 individuals showing greater immunological reactivity (including greater production of antibodies after vaccination) than individuals with Hp1-1 and Hp2-1 phenotypes(Langlois & Delanghe, 1996; S. S. Nevo & Sutton, 1968). In addition, haptoglobin has anti-inflammatory properties thus inhibits prostaglandin synthesis. It is noticeable that these anti-inflammatory properties are less

pronounced in Hp2-2 individuals (Braeckman, De Bacquer, Delanghe, Claeys, & De Backer, 1999; Langlois & Delanghe, 1996).

In the presence of haptoglobin which is considered a powerful suppressor of lymphocyte function, lymphocytes are unable to proliferate in the presence of the mitogens phytohemagglutinin and concanavalin A (Baseler & Burrell, 1983). The immune response is induced and regulated by T helper (Th) subsets specifically Th1 (cellular response) and Th2 cells (humoral response). The production of IL-2 and interferon gamma (IFN-γ) by Th1 cells induces strong IgG responses, thus favoring the cellular immune response. On the other hand, Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 increasing IgE production, thereby mediating a predominantly humoral and eosinophilic response Hp plays a role in modulating the balance between Th1 and Th2 lymphocytes by mainly promoting Th1 cell response(M. Arredouani et al., 2003). Both Th subsets are more effective in protecting against infections involving intracellular parasites and inhibit the release of Th2 cytokines responsible for defense against extracellular microorganisms. (M. S. Arredouani et al., 2005) reported that Hp modulates the inflammatory response by suppressing the synthesis of TNF-α, IL-10 and IL-12 by Lipopolysaccharides stimulated monocytes. Recent research by Guetta *et al.* (2007), correlated the increased production of cytokines (IL-6 and IL-10) by Hp1-1-Hb complex formation as compared to Hp2-2-Hb. The release of these cytokines is dependent on the binding of the Hp-Hb complexes to the CD163 receptors on macrophage. Based on this, it was suggested that Hp1-1 is more protective against vascular damage than Hp2-2 (Guetta, Strauss, Levy, Fahoum, & Levy, 2007).

1.12.5. Angiogenesis

Angiogenesis occurs under normal (healthy) as well as pathological conditions. Haptoglobin has the ability to stimulate angiogenesis (serum angiogenic factor) and promote endothelial cell proliferation and differentiation (Dobryszycka, 1997). Hp is also involved in cell migration and restructuring of the arterial walls (de Kleijn et al., 2002). The ability of haptoglobin in stimulating tissue repairs and promoting angiogenesis in ischemic conditions is useful for treating chronic inflammatory conditions. It is noteworthy that Hp2-2 is more antigenic than the other Hp phenotypes (Cid et al., 1993) .

1.13. Haptoglobin Phenotyping

The levels of Hp in human are generally stable but can change with age. Some variations in Hp concentrations are reported in the following decreasing order Hp1-1 > Hp2-1 > Hp2-2 (Imrie et al., 2006; Langlois & Delanghe, 1996). The level of Hp in fetuses, cord blood and neonates is usually very low. Investigation on ahaptoglobinemia among Brazilian neonates was found in only 8% with Hp1-1 as the predominant phenotype (de Azevedo, Salzano, & Azevedo, 1974).

Different methods are usually used to determine the concentrations of haptoglobin levels in plasma. Initially the ability of plasma to bind Hb was used. The radialimmunodiffusion method is the simplest quantifying technique for Hp (Mancini, Carbonara, & Heremans, 1965). Currently, the immunonephelometric and immunoturbidimetric assays are widely used to quantify Hp (Ramakers & Kreutzer, 1976).

Methods for haptoglobin phenotyping have followed an electrophoretic separation of the different phenotypes based on their molecular size and mobility in an appropriate gel medium

(Santoro, Boccazzi, & Carbonara, 1982). Starch gel electrophoresis was first developed in 1955 by (Smithies, 1955). This method was able to separate and specifically identify the three major Hp phenotypes. This method relied mainly on staining the separated phenotypes by peroxidase staining based on the order of their mobility in the starch gel, Hp1-1 is the smallest and fastest to migrate forming a single band. Hp2-2 protein is slowest and forms distinct large bands. However, the Hp2-1 phenotype forms a series of bands located between the Hp1-1 and Hp2-2 bands (Langlois & Delanghe, 1996). Polyacrylamide gel electrophoresis (SDS-PAGE) is another method that can be used for Hp phenotyping. Acrylamide Gel give higher resolution and can be used instead of starch gels (Peacock, Bunting, & Queen, 1965). Molecular methods, such as the polymerase chain reaction (PCR), have been recently used to genotype haptoglobin (N. S. Levy et al., 2013; Yano et al., 1998) Genotyping methods have advantages over other methods for their high specificity and sensitivity thus can detect minute amounts of circulating haptoglobin (J. R. Delanghe & De Buyzere, 2004) .

1.14. Association of Hp Polymorphism with diseases

Genetic polymorphism of haptoglobin and associated function has triggered further investigations to determine the influence of Hp subtypes in different human pathologies (Carter & Worwood, 2007). Haptoglobin polymorphism plays a significant role in parasitic, bacterial, and viral infections. Although the effect of haptoglobin polymorphism in many infectious conditions, but this effect is not always predictable due to the multifunctional character of the plasma protein (e.g., antibody-like properties, immunomodulation, iron metabolism) (Kasvosve, Speeckaert, Speeckaert, Masukume, & Delanghe, 2010). Several studies illustrated the correlation between haptoglobin phenotype and different kind of disease. There is a strong

association between different haptoglobin phenotypes and the types of diseases such as cardiovascular disease, autoimmune disorders and malignancy. Certain types of haptoglobin present can provide protection from the development of these diseases (Wassell, 2000). Hp 2-2 phenotype has been associated with atherosclerosis in hypertensive patients. High levels of this phenotype has been found in younger patients (<45 years) and in patients with previous myocardial infarction (J. Delanghe et al., 1997). The Hp polymorphism has also been implicated with iron status (Langlois et al., 2000). It was reported that Hp 2-2 is associated with increased levels of iron, ferritin and transferring saturation in males. In addition, elevated serum ferritin was associated with higher intracellular concentration in monocytes and macrophages (Langlois et al., 2000)

In DM, increased oxidative stress is common among diabetic patients resulting in glucose oxidation and modification of low-density lipoproteins (LDL). Such changes may stimulate the production of inflammatory cytokines which is implicated in morphological and pathological changes found in macrovascular and microvascular complications (Giugliano, Ceriello, & Paolisso, 1996).

Research has determined that certain Hp phenotypes and their different antioxidant properties have different effects on the degree of susceptibility to develop vascular problems in diabetic patients. Individuals with Hp 1-1 show better protection against DM than Hp 2-1 and Hp 2-2 individuals. This variation in oxidative capacity among Hp phenotypes is not associated with their Hb binding affinity; rather it is attributed to structural variations and the smaller size of Hp1-1 enabling it to pass more easily through the endothelial barrier to reach extravascular

spaces. Therefore, Hp 1-1 individuals are better protected against oxidative stress than Hp 2-1 and 2-2 individuals (Asleh et al., 2012; A. P. Levy et al., 2002; Vardi, Blum, & Levy, 2012).

The Hp phenotypes have been investigated and compared in patients with Type 2 DM patients and macro vascular complications and in normal individuals (A. P. Levy et al., 2002). It has been found that diabetic patients who carried the Hp2-2 phenotype were five times more likely to have cardiovascular complications as compared to those with the Hp1-1 phenotype. The risk of developing cardiovascular disease in these patients was intermediate in patients with Hp2-1 phenotype. These results are in agreement with reports indicating the protective role of the Hp1-1phenotype against restenosis after coronary stent implantation in diabetic patients (Roguin et al., 2002).

Research conducted by Suleiman *et al.* (2005) reported that diabetic patients with acute myocardial infarction have demonstrated that the Hp1-1 phenotype was associated with smaller infarct size and lower mortality rates at 30 days (Suleiman et al., 2005).

Arterial elasticity indices were found to be significantly lower and the vascular resistance are higher in diabetic patients who carried the Hp2-2 phenotype as compared to patients who carried Hp2-1 and Hp1-1 phenotypes (Shor et al., 2007). Such findings indicate the presence of a major predisposition to the development of atherosclerosis in homozygotes for the Hp2 allele.

It has been found that microvascular complications in both types of DM has been associated with the Hp2-2 phenotype (Nakhoul, Miller-Lotan, Awaad, Asleh, & Levy, 2007). Previously, Nakhoul *et al.*(2000) reported that the Hp2-2 phenotype is overrepresented among Israelis with Type 1 DM patients who has diabetic retinopathy while Type 1 and Type 2 DM patients carrying

the Hp1-1 phenotype had greater protection against diabetic nephropathy (Nakhoul et al., 2000; Nakhoul et al., 2001). Similar findings has been reported by Bessa *et al.* (2007) for Egyptian patients with Type 2 DM (Bessa, Hamdy, & Ali, 2007). Koda *et al.* (2002), however, did not find this protective effect attributed to the HP1 allele among patients with Type 2 DM in Japan (Koda et al., 2002).

Research has been conducted for many years to find an association between Hp phenotypes and heart disease. Reports indicated the presence of significant increase in the incidence of the Hp 2-2 phenotype in high-risk cardiac patients compared to the normal control group (Gogishvili, Kavtaradze, Mamaladze, Arutiunova, & Takadze, 1985). In 1982, Chapelle *et al.* found that the severity of damage post myocardial infarction was greater in patients with Hp2-2 than in patients carrying the Hp1-1 or Hp2-1 phenotypes (Chapelle, Albert, Smeets, Heusghem, & Kulbertus, 1982). Additionally, in patients with the Hp 2-2 phenotype who underwent a coronary artery bypass graft, the survival time was shorter than for patients with other Hp phenotypes. This can be due to the accumulation of atherosclerotic lesions in essential hypertension (J. Delanghe et al., 1997).

It has been established that the Hp 2-2 phenotype is an independent genetic risk factor for the development of coronary atherosclerosis (Stein & McBride, 1998; Van Vlierberghe et al., 2004). The reason for that could be due to the inability of this phenotype to provide protection against oxidative stress in arteries of patients with atherosclerotic plaques. It has also been considered to be a risk factor for developing refractory hypertension; therefore these patients with this phenotype require more complex antihypertensive drugs to control their blood pressure (J. R. Delanghe et al., 1995). In addition, individuals with the Hp2-2 phenotype have higher serum

cholesterol levels compared with individuals carrying other Hp phenotypes (Braeckman et al., 1999). This indicates that the different functions of Hp may be used to predict the susceptibility to cardiovascular disorders and patient prognosis.

In literature, reports have correlated specific Hp phenotypes with cancer (Bartel, Elling, & Geserick, 1985). The Hp 1-1 has been shown to be more prevalent in women with breast tumors (Awadallah & Atoum, 2004). They concluded that the Hp 1 phenotype is more common among familial cases while the Hp2 phenotype is not. Furthermore, it has been found that the Hp1 gene is overrepresented in cases with ovarian carcinoma (Dobryszycka & Warwas, 1983). However, an association between the Hp2-1 phenotype and a family history of ovarian carcinoma has been reported (Frohlander & Stendahl, 1988).

It is apparent that each Hp phenotype is more common with certain malignancies. The Hp 2-2 for example has been found in lower frequency in patients with pulmonary adenocarcinoma and bladder carcinoma than in normal controls(Beckman, Eklund, Frohlander, & Stjernberg, 1986) On the other hand, the Hp2-1 and Hp2-2 phenotypes were found in significantly higher frequency in patients with esophageal and gastric cancers(Jayanthi et al., 1989).

The correlation between Hp phenotypes and different types of leukemia has also been investigated. The Hp 1-1 phenotype has been detected in patients with acute lymphatic (ALL), acute myeloid (AML) and chronic myeloid (CML) leukemia (S. Nevo & Tatarsky, 1986). However, Frohlander (1984) did not observe any association between Hp phenotypes and leukemia but ahaptoglobinemia was significantly more frequent in these patients (Frohlander, 1984). In another study, a low frequency of Hp2-2 has been observed in patients with IgA myeloma (Germenis, Babionitakis, Kaloterakis, Filiotou, & Fertakis, 1983).

It has been demonstrated in literature that Hp polymorphism may play a role in a number of infectious diseases. One of the known functions of haptoglobin is the prevention of bacterial growth by removing free iron. This natural bacteriostatic property has affected the growth of some bacterial pathogens such as *Neisseria meningitides*, *Campylobacter jejuni* and *Bacteroides fragiles* (Eaton, Brandt, Mahoney, & Lee, 1982; Lewis & Dyer, 1995; Pickett, Auffenberg, Pesci, Sheen, & Jusuf, 1992). Earlier studies showed that haptoglobin provided protection to rats that has been inoculated with pathogenic *Escherichia coli* and Hb (Eaton et al., 1982). In cases with tuberculosis, it has been reported that the death rate was 6.1 times higher in patients who carried the Hp2-2 phenotype than those with the Hp 1-1 (Kasvosve et al., 2000).

In HIV viral infections, cell to cell viral transmission is mediated by families of integrin adhesion receptors such as CD11a-c and CD18 which was found to contribute to variations in the survival rates. In HIV-seropositive patients, the circulating plasma iron could enhance Hb-driven oxidative stress which favors viral replication and transmission. Delanghe *et al.*in 1998 reported a correlation between HIV-seropositive patients with Hp2-2 phenotype and higher mortality and worse prognosis than patients with other phenotypes (J. R. Delanghe et al., 1998).

Chronic hepatitis C infections has been associated with Hp polymorphism (Hp1-1 was overrepresented)(H. K. Louagie et al., 1996). Such finding may indicate that Hp phenotype may affect the clinical evolution of hepatitis C. Interestingly; Hp2-2 individuals develop lower levels of antibodies after vaccination against hepatitis B than those with Hp1-1 or Hp2-1 phenotypes(H. Louagie et al., 1993) .

In Sudanese patients infected with *Plasmodium falciparum,* significant increase in the levels of Hp1-1 in patients with uncomplicated and complicated forms of the disease has been reported

(Elagib, Kider, Akerstrom, & Elbashir, 1998). The phenotype frequency among these patients was 60.8% for Hp1-1, 29.7% for Hp2-1 and 9.5% for Hp2-2. In healthy (control) individuals from the same region, phenotype frequency was 26.0%, 55.8% and 18.3%, respectively. Hp1-1 phenotype was also predominant among patients with severe *P. falciparum* malaria living in the coastal region of Ghana (Quaye, Brandful, Ekuban, Gyan, & Ankrah, 2000). Consistent with these reports associating the prevalence of Hp1-1 with *P. falciparum* infections, (Minang et al., 2004) found that the Hp1-1 phenotypes had higher prevalence in placental infection by *P. falciparum* in pregnant women at delivery in western Cameroon.

1.15. Objectives and Specific Aims:

Haptoglobin polymorphism has not been researched or addressed in Palestine. There is a wealth of literature relating the haptoglobin phenotypes with various conditions, diseases and infections. All pathogenic microorganisms have developed mechanisms for scavenging iron from circulation. The association of pathogenicity of microorganisms and iron has been repeatedly reported in literature. Haptoglobin polymorphism has been reported to play an important role in infectious diseases and/or protection from it.

Therefore, this study has been designed to evaluate the relationship between nasal carriage of *S. aureus* and the different haptoglobin phenotypes among college students at Birzeit University. This study is the first one conducted to determine the specific haptoglobin phenotype in *S. aureus* nasal carriage among Palestinian population. The specific aims of this project were:

i. To determine the rate of nasal carriage among first year college students at Birzeit University.

ii. Determine the allele frequency of haptoglobin among the Palestinian population.

ii. Determine the haptoglobin polymorphism among *S. aureus* nasal carriers and compare it with normal population or non-carriers.

Chapter 2

Materials and Methods

2.1. Sample collection and samples information

2.1.1. Nasal swabs and processing

A total of 1500 nasal swabs were collected during the month of September 2012 and August – October 2013 from 18-20 year old newly admitted students at Birzeit University (Birzeit, Palestine). The nasal swabs were collected according to standard procedure and screened for *S. aureus* nasal carriage. A signed consent was obtained from each student prior to enrollment in the study.

The nasal swabs were immediately inoculated on mannitol salt agar (Oxoid, England) and incubated at 37^{o}C for 24 hours. Mannitol fermenting Gram positive, Catalase positive bacteria were primarily considered S. *aureus.* Definitive confirmation of the identity of the isolates was made by the tube coagulase test (bound and free) and the Staphylase test.

2.1.2. Blood samples

Blood was collected in EDTA containing tubes from all participants by the peripheral vein puncture procedure following aseptic techniques. CBC, Blood grouping and Rh typing were done on all samples. Plasma was separated by centrifugation of the EDTA tubes at 3000 rpms for 15 minutes, separated plasma were stored at -20°C for haptoglobin phenotyping.

2.2. Starch Gel Electrophoresis of Haptoglobin

Starch gel electrophoresis is a simple method used for separation and phenotyping of proteins and haptoglobin. Starch is safe and considered a simple and useful method for protein electrophoresis. A characteristic feature of starch gels is that they exhibit molecular sieving effects. Separation occur based on differences in charge, size and shape of the proteins (Na et al., 2006). For haptoglobin electrophoresis, 12% (w/v) starch gel slabs were prepared with a thickness of 8 mm. The starch gels were prepared by dissolving the starch in Tris-citrate buffer pH 8.6. Borate buffer was used as the running gel. The staining solution was made in 0.1 M acetate buffer.

Following is the preparation of the different buffers and staining reagent used in the starch gel electrophoresis conducted in our experiments:

Buffers and reagents

1. Tris-Citrate Buffer (Gel buffer)

Citric acid	1.05 g
Tris base	9.19 g
H2O	1 liter

Adjust pH to 8.6 with HCL.

2. Borrate buffer (Running Buffer)

Boric acid 18.6 g

NaOH 3.12 g

H2O 1 liter

Adjust pH to 8.4 with HCl.

Staining Solution

3-amino-9-ethylcarbazole/N,N-dimethylformamide(AEC/DMF dye) is prepared as follows:

1. AEC/DMF dye 40 mg AEC + 10 ml DMF

2. Solution A 11.55 ml glacial acetic acid/L of dH2O

3. Solution B 16.4 g Na-acetate/L of dH2O

4. Mix 22.3 ml of solution A with 30 ml solution B and add enough dH2O make a total of 200 ml and then add the AEC/DMF prepared in step 1.

5. Add 4 ml of 30% H2O2 to the staining solution in step 4.

6. The solution is ready to add to the gel for staining for one hour at 37^{o} C.

7. 5% glacial acetic acid was added to the stained gel.

2.3. Methods

2.3.1. Preparation of Starch Gels

Starch gels were prepared at 12% concentration by mixing 30g of dry starch (Sigma) in250 ml Gel Buffer (pH 8.6). The suspension was heated on the flame of a Bunsen burner with continuous swirling and vigorous agitation until it became thickened and less viscous. The hot starch solution was then degassed and poured into the center of the plate Figure 10 and allowed

to spread over the surface. The plate was then left for 30 minutes at room temperature to allow the starch solidify and polymerize. After solidification, the gel plate was transferred to a refrigerator at 4^{o}C and kept for 2-4 hours.

2.3.2. Preparation of Hemolyzed Red Blood Cell Suspension

Whole blood was collected in EDTA lavender top tubes prior to use. A total of 2 ml whole blood was then washed three times with normal saline and centrifuged at 3,000 rpms for 5 minutes each time. The sediment and washed red cells were lysed by adding 2 ml distilled water, vortexed and centrifuged at 13,000 rpms for 5 minutes, the lysed cells were then aliquoted in 0.5 ml and used for 2 or 3 successive days.

2.3.3. Hemoglobin-Haptoglobin Complex Formation

A total of 30ul of plasma from each sample was mixed with 5 ul of the freshly hemolyzed red blood cell suspension and incubated at room temperature for 10 minutes. The hemoglobin-haptoglobin complex is now ready for starch gel electrophoresis. The solidified starch gel was cut with a blade approximately 3-4 cm from the cathode . An aliquot of 15-25 ul of fresh prepared Hb-HP complex was applied to Whatman filter paper that is 6 mm thick and cut in 0.3 X 0.5 cm pieces. The soaked Whatman strips were then inserted into the incision made in the gel and spaced 2-3 mm apart Figure 10.

2.3.4. Electrophoresis

The starch gel plates with the loaded samples were placed on the platform of the electrophoresis apparatus. To prevent evaporation, the gel was covered with thin plastic membrane. Three layers of filter paper were placed on each side of the starch gel with the other end placed in the running buffer. Electrophoreses was conducted for 2-4 hours at200 to 300 volts/cm and 30 – 40 mA until a migration of the complexes reaches 3 -4 cm away from the anode. The gel set up used is shown in Figure 9, 10.

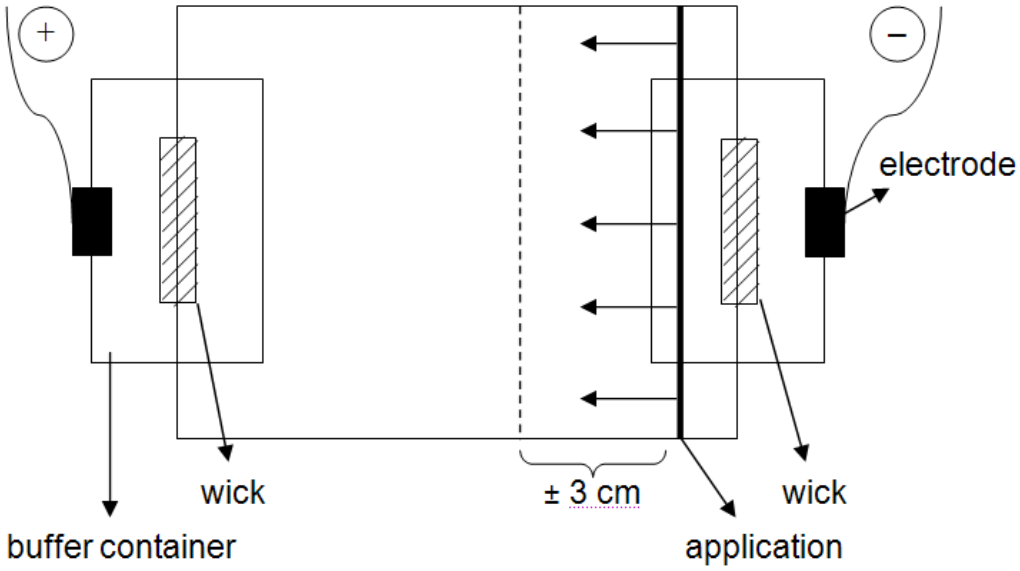

Figure 9: Schematic diagram illustrate Starch gel electrophoresis technique

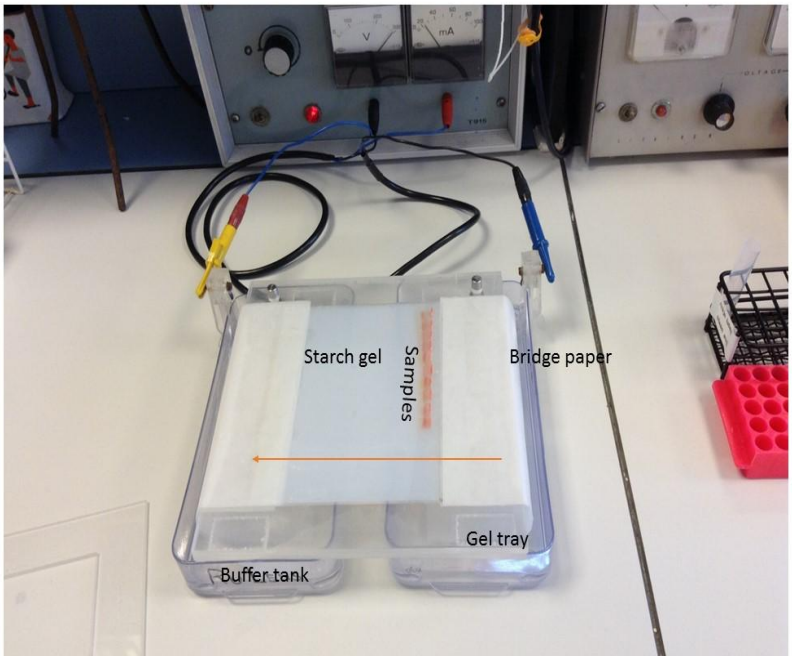

Figure 10: Starch gel apparatus.

2.3.5. AEC Immunoperoxidase Staining

After electrophoresis, the top layer of the plate frame and wicks were removed. The gel was then cut into two layers of different thickness using fishing thread. The two layers (6 mm thick and 3mm thick gels) were stained by immunoperoxidase stain as follows:

- Pour the staining mix (200 ml) to the gel in a staining tray.
- Place on an oval rocker with gentle agitation.
- Leave the gel with the staining solution for one hour at 37^{o} C.
- Remove the staining solution and discard in a proper container.
- Stop the staining by adding 5% Acetic acid (200 ml) solution.

Interpretation of the gel

After staining the starch slices of the gel, few banding pattern can be observed as shown in Figure 11 . The fastest or more rapidly migrating band is the free haemoglobin. Free haemoglobin is followed by a band for the haptoglobin–haemoglobin complex . The banding patterns for the different haptoglobin phenotypes as shown in Figure 11 are interpreted as follows:

- Hp1-1 shows single large banding close to the free haemoglobin band.
- Hp2-1 shows several small banding pattern trailing behind the free hemoglobin front.
- Hp2-2 shows a characteristic polymeric pattern of the largest and slowest band pattern distinct from the haemoglobin front band.

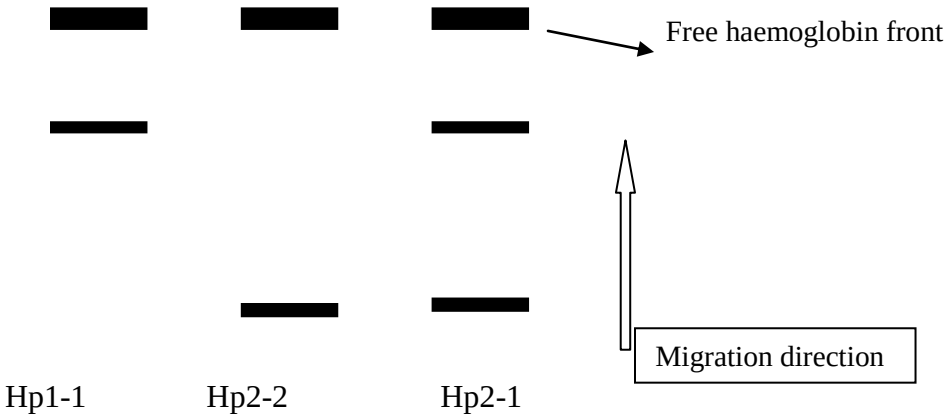

Figure 11: Electrophoresis pattern of Haptoglobin phenotypes.

2.4. Allele Frequency Calculation:

Allele frequencies of the haptoglobin genes were determined by gene counting and the goodness of fit of the genotype distribution according to the Hardy-Weinberg equilibrium.

2.5. Statistical Analysis

Data were statistically analyzed using SPSS (Statistical Package for Social Science) (version 17; Inc., Chicago. IL). The qualitative data were expressed as frequency and percent, One way analysis of Variance (ANOVA) test was used for comparison between three or more groups Chi-square (X2) test was used to compare the qualitative variables. The allele frequencies were calculated for a two-allele system and differences in haptoglobin genotype and subtype frequencies between groups were compared using (X2) test. P-value was considered statistically significant when it is less than 0.05.

Chapter 3

Results

3.1. *Staphylococcus aureus* nasal carriage rate

This study was conducted on nasal swabs collected from a total of 1500 first year college students admitted at Birzeit University (Birzeit, Palestine) between 2012 and 2014.The nasal swabs were cultured and nasal carriage of *S. aureus* was determined. Furthermore, whole blood was collected from each participating student to determine the prevalent phenotypes of haptoglobin in *S .aureus* carriers and controls.

As shown in Figure 12, Nasal carriage was seen in 21.5% (324/1500). Haptoglobin phenotyping was determined for 224 *S. aureus* carriers and 99 controls. The results for the nasal carriage and haptoglobin phenotypes are shown in the following Figure 12 and Table3

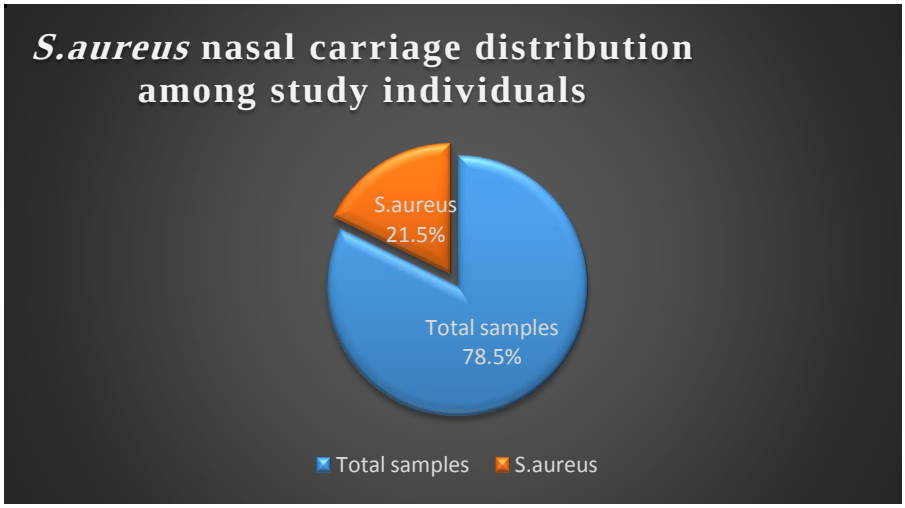

Figure 12: Rate of *S. aureus* carriers in the total sample.

Table 3: Rate of S. *aureus* nasal carriage in the al sample collected.

	No. of positive	Percent
S.aureus carrier	323/1500	21.6%
Control	1177	78.4%
Total	1500	100%

3.2. Haptoglobin polymorphism among cases and control

Haptoglobin phenotyping was done on 323 samples using starch gel electrophoresis. Haptoglobin polymorphism was determined for 269 samples. The remaining 54 samples gave un-interpretable results. The starch gel electrophoresis results found for Hp polymorphism is shown in Figures 13. Figure 13 shows the electrophoresis patterns of the haptoglobin proteins according to their genotypes. Hp 1-1 shows a fast migrating band corresponding to the small Hp 1 dimer, and Hp 2-2 displays multiple slow migrating bands representing polymers consisting of Hp 2 proteins. Hp 2-1 has a fast migrating band and several slow migrating bands.

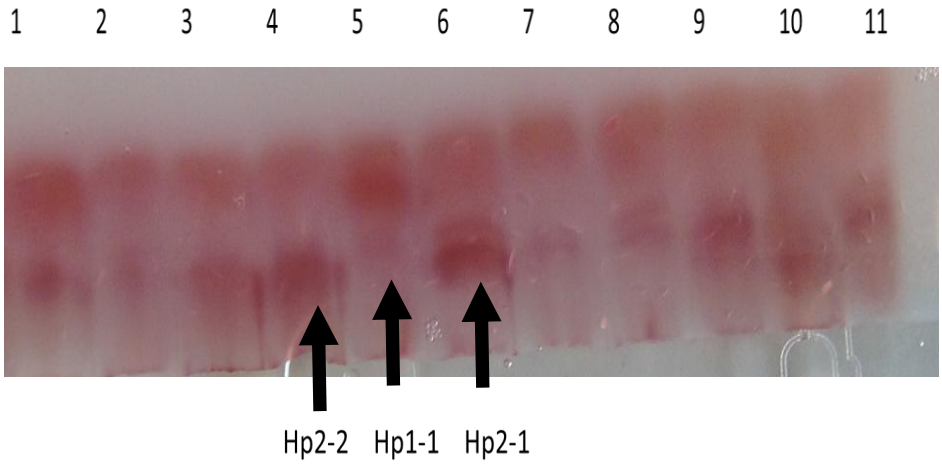

Figure 13: Starch gel electrophoresis showing the various phenotypes of haptoglobin.

The Hp phenotypes have been determined for *S. aureus* carriers (163 females and 61 males) and controls (77 females and 22 males). Phenotyping for the majority of the samples has been conducted under the supervision of Dr. Professor Joris Delanghe in his laboratory at Gent University Hospital, Belgium. The remaining sample has been analyzed in the Molecular Diagnostic Center in Birzeit University with continuous consultation with Professor Dr. De. Delanghe and Dr. Mohammad Farraj. The results are shown in Figure 14. The results shown in the figure can be summarized in the high expression of Hp 2-1 in the controls. It is also apparent among the control group that the rate of Hp 2-2 phenotype is lower than Hp 2-1. On the other hand, the carrier group has consistently higher frequency of Hp 2-2 than the other phenotypes. Hp 2-1 phenotype has been less expressed among carriers as compared to the controls. Hp 1-1 phenotype has the least expression among the female control group.

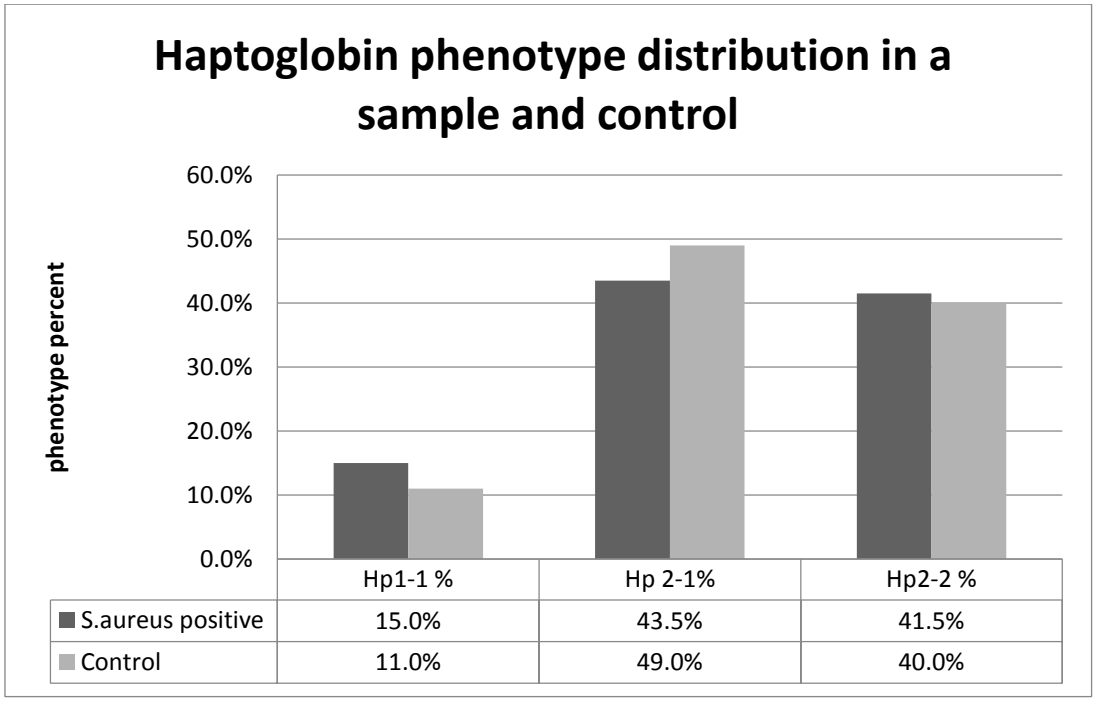

Figure 14: Haptoglobin phenotype among *S. aureus* carrier and control population

The results of haptoglobin polymorphism for *S. aureus* carriers and the control group are summarized in Table 4. Hp1-1 frequency was 12% in *S. aureus* carriers and 10% in the control group, while 34% Hp2-1 for *S,aureus* carriers and 44.4% in the control group. The frequency of Hp2-2 was 34% for *S. aureus* carriers and 35.5% for the control group. The phenotypes of 20% of the *S. aureus* carriers could not be determined using the starch gel electrophoresis method and 10% of control group as well.

Table 4 : Distribution of haptoglobin polymorphism between. *S aureus* carriers and control groups among Birzeit University students.

Haptoglobin phenotypes						
Case	Total Number	Hp 1-1 %	Hp 2-1 (%)	Hp 2-2 %	Undetectable %	P-value
***S. aureus* positive**	224	(27)12%	(77)34%	(76)34%	(44)20%	>0.05
Controls	99	(10)9.9 %	(44)44.4	(35)35.4%	(10)10%	>0.05
Total	323	37	121	111	54	

If we neglect the undetected samples in both control and case groups, phenotype frequency distribution between the two groups will change as shown in Table 5.

Table 5: Haptoglobin phenotype distribution in a sample and control without undetectable samples

Haptoglobin phenotypes				
Case	Total Number	Hp1-1 %	Hp 2-1%	Hp2-2 %
S.aureus positive	180	15%	43.%	42%
Control	89	11%	49%	40%

Haptoglobin polymorphism has been determined for the male and female participants in the study. The results are shown for both cases and controls in the Table 6 below. There was no significant difference (P>0.05) between female carriers of *S .aureus* and control group as shown in Figure 15. However, there was a significant difference (P=0.015) when *S. aureus* male carriers compared to the male control group showing higher expression of Hp2-2

Table 6: Haptoglobin polymorphism according to gender among the case and control groups.

***S. aureus* Carriers**					
Gender/Phenotype	Hp1-1 % (No)	Hp2-1	Hp2-2	Undetectable	P-value
Female s 163	10.5 (17)	38 (62)	35.5 (58)	16 (26)	NS
Males 61	16.4 (10)	24.6 (15)	29.5 (18)	29.5 (18)	0.015
Total 224	27	77	76	44	
Control					
Females 77	6.5 (5)	41.5 (32)	39 (30)	13 (10)	NS
Males 22	22.7 (5)	54.6 (12)	22.7 (5)	0	0.015
Total 99	10	34	35	10	

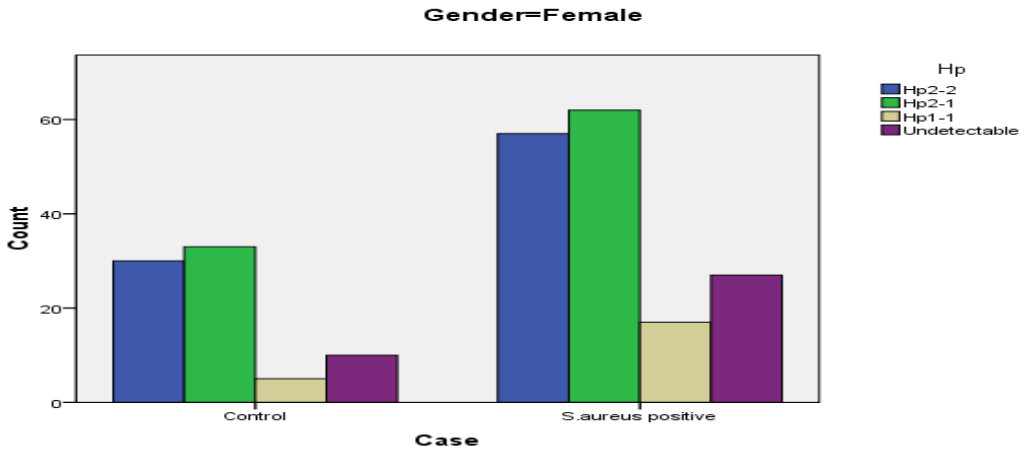

Figure 15: Different hatpoglobin phenotypes for *S.aureus* and case in female population

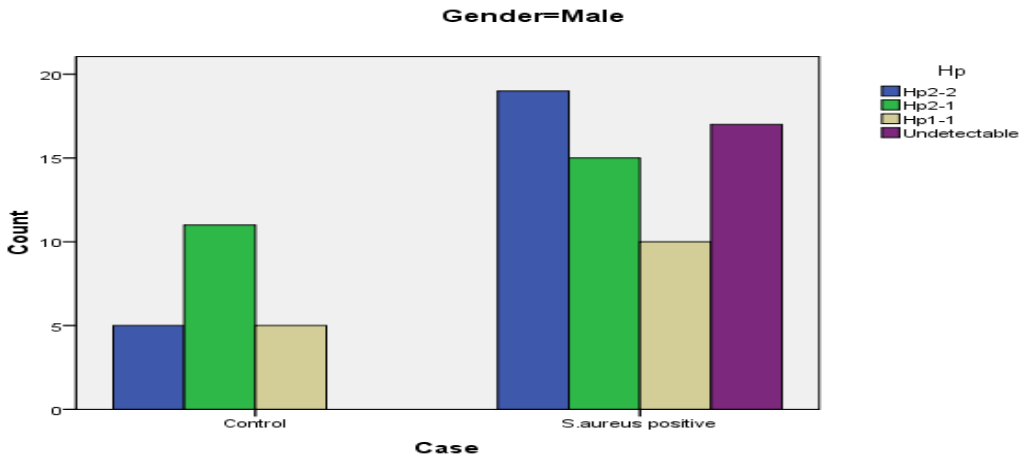

Figure 16: Different haptoglobin phenotypes for *S.aureus* and control group in male population

3.3. Allele Frequency of Haptoglobin

Allele frequency of haptoglobin has been reported to be dependent on race (Carter & Worwood, 2007). Reported Hp^1 allele frequencies among Arabs living in Israel, Saudi Arabia and Iraq were 0.36, 0.45 and 0.29 respectively as shown in Table 6 Allele frequency among Palestinian control population tested in this project who are living in the West Bank has been determined as 0.36 for

Hp^1 and 0.64 for Hp^2 allele. Allele frequency for the Palestinian population nasal carriers of *S. aureus* was similar to the control group as shown in Table 7.

Table 7: The Frequency of Haptoglobin Allele in *S.aureus* positive and Control Groups.

Hp allele	**Frequency**	
	S.aureus	Control
Hp1	0.37	0.36
Hp2	0.63	0.64

Chapter 4

Discussion

4.1. Prevalence of *S. aureus* nasal carriage among study population:

S. aureus is a commensal that colonizes several parts of the human body and mucus membranes and a common cause of nosocomial and community acquired infections as well. The anterior nares are the most frequent site for *S. aureus* colonization and commonly correlated with clinical infections by this organism (Wertheim et al., 2005) and subsequent evolution of methicillin resistant strains (MRSA). The prevalence of nasal carriage of *S. aureus* among the Palestinian population has previously been determined to be 25% to 30% (Kaibni et al., 2009). In this study, the prevalence of nasal carriage was 21.5%.The main aim of this project was not to determine the prevalence of *S. aureus* nasal carriage but to correlate it with haptoglobin polymorphism. The prevalence of nasal carriage in this study was 21.5%, lower than previously reported. This indicates better management and improved hygiene measures taken to curb the nasal carriage of this organism.

There are three different states for *S. aureus* nasal carriage that can be classified as: persistent carriage, intermittent carriage, and no carriage. This study didn't provide insight into staphylococcal carriage states of the studied population. Nouwen et al., 2004 suggested that Persistent *S. aureus* nasal carriage has a higher risk of infection, whereas intermittent carriers and non-carriers have lower risk (Nouwen et al., 2004).

Nasal carriage state can be determined by obtaining two samples one week apart with results confirming the presence of *S. aureus*. This was considered the reference procedure to accurately predict the persistent **S. aureus** carriage state with a reliability of 93.6% (Nouwen et al., 2004).

Therefore, this method should be used in studies concerning the determination of **S. aureus** nasal carriage state.

4.2. Haptoglobin phenotypes in nasal carriage of *S. aureus*:

Several studies have demonstrated the relation of Hp polymorphism with susceptibility to infections and other diseases and severity of clinical symptoms (Melamed-Frank et al., 2001). However, to the best of our knowledge through literature searches, the correlation between *Staphylococcus aureus* nasal carriage and Hp has not yet been studied .

The Hp polymorphism in the sample tested in this study has been determined for *S. aureus* carriers (163 females and 61 males) and controls (77 females and 22 males) by the starch gel electrophoresis method. In the present study, we found that the individuals carrying *S. aureus* in their anterior nares was non significantly higher with Hp 2-2 and Hp1-1 phenotypes than in control group ,suggesting that people with the Hp2-2 phenotype are more susceptible to *S.aureus* infections or colonization as compared to the other phenotypes; Hp 2-1 phenotype. According to Kristiansen et al., this may explained by the higher affinity of the Hp-Hb complex for CD163 emphasizing the notion of Hp phenotype-dependence relating *S. aureus* nasal carriage with haptoglobin polymorphism (Kristiansen et al., 2001).The results are shown in Figure 15,16 and Table 6 . The striking differences shown in the Figure can be summarized in the extremely high expression of Hp 2-1 in the controls which reflects its protective effects against infections. It is also apparent among the control group that the rate of Hp 2-2 phenotype which is correlated with higher frequency of infections is lower than Hp 2-1. On the other hand, the *S.aureus* carrier group has consistently higher frequency of Hp 2-2 than the other phenotypes which may play a protective role. It is also apparent that the Hp 2-1

phenotype has been less expressed among carriers as compared to the controls. Furthermore, there were 20% of the cases and 10% of the controls cannot be precisely be phenotyped by starch gel electrophoresis method. One interpretation for that could be the presence of Hp^{del} gene in some of the samples tested ,and Hp ^{del} could not be identified by the routine electrophoresis methods and requires further molecular identification such as polymerase chain reaction (PCR). The sensitivity of the starch gel method can also contribute to our inability to identify the haptoglobin phenotype in the undetectable samples. In addition, the overlapping size of the various phenotypes could contribute to this effect. Furthermore, low levels of haptoglobin due to hemolysis can also contribute to having this high rate of undetermined haptoglobin phenotypes in the sample tested.

Haptoglobin polymorphism according to gender indicated the presence of significant association between *S. aureus* nasal carrier males and the Hp2-2 phenotype (P= 0.015). However, this association was not detected in the female group (P>0.05).

Haptoglobin polymorphism can be associated with various diseases as well as infections. We were unable to associate haptoglobin polymorphism with *S. aureus* nasal carriage among humans. In our study, there was no significant association between *S. aureus* carriers and control group by one way ANOVA (P=0.067)

4.3. Allele frequency

Variations in allele frequency of the Hp^1 and Hp^2 genes have been reported worldwide depending on racial origin. The Hp^1 allele frequency for example varied from about 0.07 in some Indian population to over 0.7 among some West African and South American populations

(Carter & Worwood, 2007). Limited reports have been found in literature addressing allele frequency among Arabs in the Middle East and none was found for Palestinian living in west bank and Gaza strip. Reported haptoglobin allele frequency by Ramot et al. in 1961 and 1962 was limited to Arabs living in Israel, Iraq and Saudi Arabia(Carter & Worwood, 2007) . It is apparent that the allele frequency for haptoglobin among the Palestinians tested in this study represents a new finding. We were unable to find in literature the allele frequencies for Hp^1 and Hp^2 genes for Palestinian population and hence we can't compare our findings with others. However, our results are somewhat similar to allele frequency reported for the Israeli Arab population and the Jordanian population Table 1 (Carter & Worwood, 2007; Farhud, 1980). This similarity can be expected since considerable numbers of Palestinians are living in Jordan in refugee camps or integrated with the Jordanian population since the 1948 expulsion due to Israeli occupation.

Table 8: Distribution of Hp1 gene frequencies in the Middle East adopted from (Farhud, 1980).

Origin	**No. tested**	**Hp1 frequency**	**Reference**
Turkish	274	0.25	Hummel, 1970
Kuwaitis	158	0. 345	Sawhney (1975)
Iraquis	118	0. 288	Ramot et al (1961)
Iraq (Jews)	118	0. 29	Ramot et al. (1962)
Iraq (Jews)	197	0. 270	Fried et al. (1963)
Arabs (S. Arabia)	92	0. 445)	Marengo-Rowe et aI. (1974
Israeli (Arabs)	75	0. 36	Ramot et al.

			(1962)
Israelis (Orientals)	345	0. 26	Goldschmidt et al. (1962)
Israeli (Kurds)	113	0. 36	Ramot et al. (1962)
Kurdish Jews	96	0. 300	Fried et al. (1963)
Israeli (Ashkenazim)	170	0. 34	Ramot et al. (1962)
Israeli (Ashkenazim)	499	0.27	Goldschmidt et al. (1962)
Ashkenazi Jews	699	0. 300	Fried et al. (1963)
Israel (Iran Jews)	158	0. 310	Simhai (1976)
Yemen Jews	41	0. 250	Fried et al. (1963)
Haban Jews	589	0. 210	Bonn6 et al. (1970)
Towara Bedouin	198	0. 429	Bonn6 et aI. (1971)
Jebelliya Bedouin	95	0. 789	Bonn6 et al. (1971)
Iranians	627	0. 281	Present study
Iranians	360	0. 270	Farhud & Walter (1972)
Iranians	34	0.25	Harris et al. (1959)
Iranians	97	0. 354	Walter & Djahanschahi (1963)
Iranians	1,016	0. 28	Miyashita &

			Ohkura (1975)
Iranians (Caspian Littoral)	448	0. 214	Kirk et al. (1977)
Iranians	1,566	0. 288	Bajatzadeh & Walter (1969)
Iranians	l, 020	0. 305	Bajatzadeh & Walter (1968)
Iranians	275	0. 296	Sawhney (1975)
Iranian Moslems	429	0. 28	Bowman (1964)
Iranian Zorastrians	145	0. 19	Bowman (1964)
Iranian Ghashquai	117	0. 33	Bowman (1964)
Iranian Jews	91	0.30	Ramot et aI. (1962)
Iranian Jews	101	0. 290	Fried et al. (1963)
Iranian Jews	459	0. 320	Tabatabai (1976)
Iranian Armenians	228	0. 344	Tabatabai (1976)

Recommendations

Looking into the outcomes of this project, we recommend to expand the study to include a larger population and collect a more representative sample accounting for age, gender, type of disease among other things. Determination of *S. aureus* nasal carriage state would add value to the project and provide important information to healthcare providers. Additionally, it is important to evaluate the antimicrobial susceptibility profiles for the tested *S. aureus* isolates and associate that with haptoglobin polymorphism. Since this project is mainly concerned about the outcome

of the battle for iron between the body defense mechanisms and the scavenger pathogens, it would be very useful and informative to determine the iron status of the study population.

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Attachment I

برنامج الماجستير في العلوم الطبية المخبرية
موافقة على المشاركة في البحث

عنوان البحث:

Haptoglobin polymorphism among *S. aureus* nasal carriage

Birzeit University students.

تعدد اشكال الهابتوجلوبين بين طلبة جامعة بيرزيت الحاملين لبكتيريا المكورات العنقودية المذهبة في الانف

الباحثون: د. محمد فراج، منذر ميتاني/ جامعة بيرزيت

حيثيات ألبحث: نرغب بمشاركتكم في هذا البحث.من خلال الموافقة على أخذ كمية قليلة من الدم الذي تم الحصول عليه من قبل عيادة الجامعة لعمل الفحوصات الروتينية لطلبة جامعة بيرزيت. واخذ مسحة من الانف ولمساعدتكم في اتخاذ القرار نود أن نوضح ما يلي:

1. الغرض والإجراءات المتبعة في البحث: لدراسة مدى انتشار MRSA وتحديد نسبة الحاملين لهذه البكتيريا.
2. أية مخاطر يمكن نوقعها لا توجد مخاطر عدا عن الحد الأدنى من الراحة أثناء سحب الدم واخذ مسحة الانف.
3. كيف سيتم الحفاظ على السرية: لن يتم استخدام اسم المشارك ، سيتم ترميز العينة.
4. أي علاج طبي في حالة وجود نقص في أي من الفحوصات : يتم إبلاغ الشخص بالنتيجة والمتابعة مسؤوليته الشخصية.
5. عدد ألمشاركين : الطلبة الجدد – سنة اولى

إذا وافقت على المشاركة، سوف تحصل على نسخة موقعة من هذه الوثيقة. ويمكنك الاتصال بالمختبر على 2505 للإجابة على أي استفسار حول نتائج الفحص الخاص بك .

ان مشاركتك في هذا البحث سوف تسهم في زيادة المعرفة. ونتائج هذه الدراسة سيكون لها تأثير ايجابي على الوضع الصحي في المجتمع الفلسطيني ولهذا نشكرك جدا على المشاركة.

توقيع المشارك: _____

التاريخ: _____

Attachment II

Statistical significance;

Statistical analysis was performed by Chi square and one way ANOVA using SPSS 17 statistical package. The results of the analysis and the significant association between the variables are shown in the following Tables and Figures

Statistical analysis

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Case * Hp	323	100.0%	0	.0%	323	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.866[a]	3	.118
Likelihood Ratio	6.185	3	.103
Linear-by-Linear Association	4.184	1	.041
N of Valid Cases	323		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.34.

Symmetric Measures

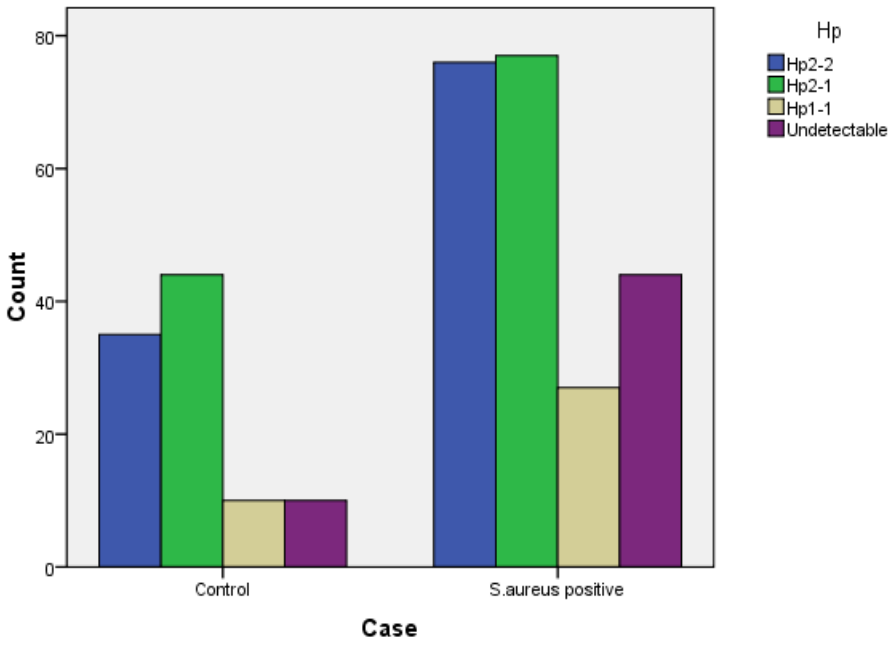
		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Nominal by Nominal	Phi	.135			.118
	Cramer's V	.135			.118
Interval by Interval	Pearson's R	.114	.049	2.056	.041[c]
Ordinal by Ordinal	Spearman Correlation	.079	.053	1.412	.159[c]
N of Valid Cases		323			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

c. Based on normal approximation.

Bar Chart



Case Processing Summary

	Cases

	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Case * Hp * Gender	323	100.0%	0	.0%	323	100.0%

Chi-Square Tests

Gender		Value	df	Asymp. Sig. (2-sided)
Female	Pearson Chi-Square	1.838[a]	3	.607
	Likelihood Ratio	1.909	3	.592
	Linear-by-Linear Association	.894	1	.344
	N of Valid Cases	241		
Male	Pearson Chi-Square	10.415[b]	3	.015
	Likelihood Ratio	14.221	3	.003
	Linear-by-Linear Association	4.991	1	.025
	N of Valid Cases	82		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.12.

b. 2 cells (25.0%) have expected count less than 5. The minimum expected count is 3.84.

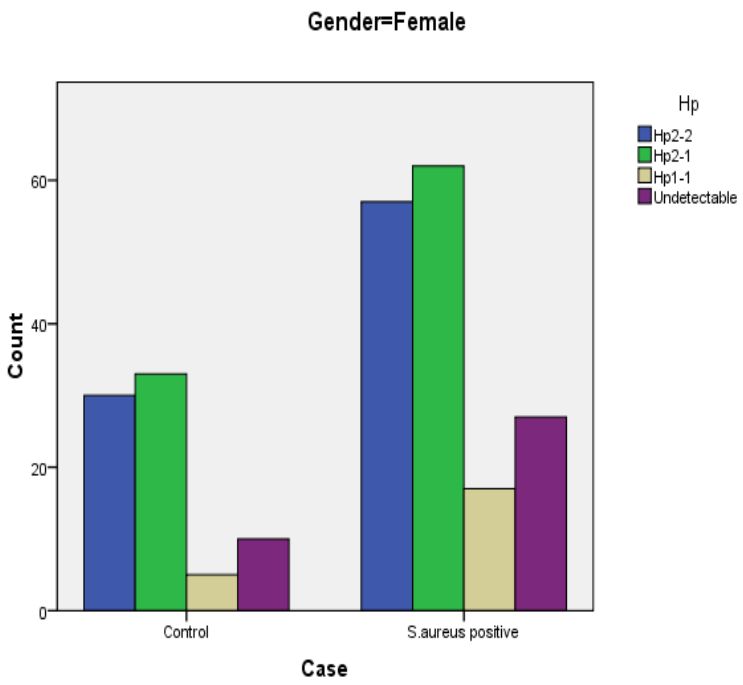
Symmetric Measures

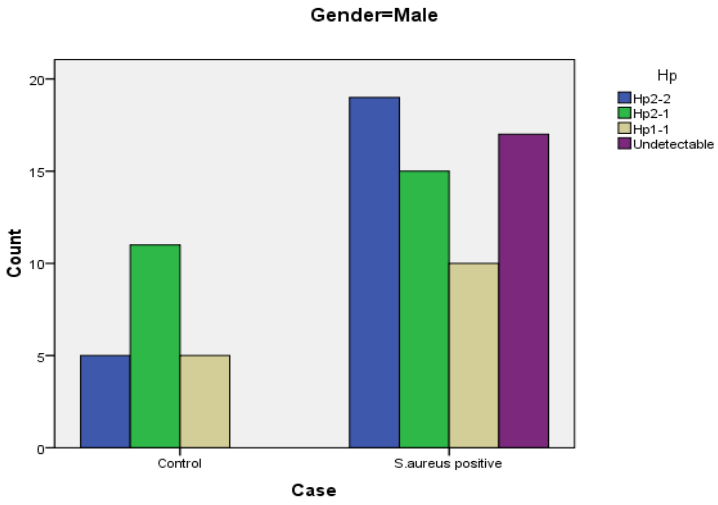
Gender			Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Female	Nominal by Nominal	Phi	.087			.607
		Cramer's V	.087			.607
	Interval by Interval	Pearson's R	.061	.062	.945	.345[c]
	Ordinal by Ordinal	Spearman Correlation	.063	.063	.976	.330[c]
	N of Valid Cases		241			
Male	Nominal by Nominal	Phi	.356			.015
		Cramer's V	.356			.015
	Interval by Interval	Pearson's R	.248	.059	2.292	.025[c]
	Ordinal by Ordinal	Spearman Correlation	.130	.093	1.177	.243[c]
	N of Valid Cases		82			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

c. Based on normal approximation.


Gender=Female
Hp
Hp2-2
Hp2-1
Hp1-1
Undetectable
Count
60
40
20
0
Control
S.aureus positive
Case


Gender=Male
Hp
Hp2-2
Hp2-1
Hp1-1
Undetectable
Count
20
15
10
5
0
Control
S.aureus positive
Case

One way ANOVA analysis foe *S .aureus* sample compared with control sample according to gender

ANOVA

		Sum of Squares	df	Mean Square	F	P value
Case	Between Groups	.279	1	.279	1.311	.253
	Within Groups	68.377	321	.213		
	Total	68.656	322			
phenotype	Between Groups	.400	1	.400	3.389	.067
	Within Groups	31.511	267	.118		
	Total	31.911	268			

ANOVA test revealed no significant difference between nasal carriers and control group with Gender. P o0f <0.05 is significant