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Staphylococcus aureus nasal carriage, capsular polysaccharides serotypes and host immune response in End Stage Renal Disease patients

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دراسة العلاقة بين حمل مرضى غسيل الكلى لبكتيريا المكورات العنقودية الذهبية في الأنف و رد الفعل المناعي و ألنمط المصلي لهذه البكتيريا

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This thesis is dedicated to my mother, brothers and sisters, without whom it would never have been accomplished .

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Abbreviations:

AVH:	Augusta Victoria Hospital
CP5:	Capsular polysaccharides type 5
CP8:	Capsular polysaccharides type 8
C3:	Complement 3 fragments
DM:	Diabetes mellitus
ESRD:	End Stage Renal Disease
FBP:	Fibrinogen binding protein
FnBPs:	Fibronectin-binding proteins
HIV:	Human immune deficiency virus
HTN:	Hypertension
LTA:	Lipoteichoic Acid
MRSA:	Methicillin Resistant *S. aureus*
MSA:	Mannitol Salt Agar
NCDs:	Non-Communicable disease
NESRD:	Non-End Stage Renal Disease
PG:	Peptidoglycan

Abstract

Staphylococcus aureus nasal carriage among patients on hemodialysis is considered a risk factor for endogenous *S. aureus* vascular-access infection. During the period from May 2004 to January 2005, 99 end stage renal disease patients at Ramallah hospital, Augusta Victoria hospital, and Alia hospital were screened for nasal carriage of *S. aureus* and 41 (41.4%) were colonized by *S. aureus* . For the follow-up of the nasal carriage pattern, periodic nasal swabs were obtained and three *S. aureus* nasal carriage pattern have been distinguished: persistent (17.17%), intermittent (24.24%), and non-carrier (58.6%). Most of the *S. aureus* clinical isolates possessed capsular polysaccharides of multiple serological types, (48.15%) possessed a polysaccharide type 5 capsule, (33.33%) possessed a polysaccharide type 8 capsule, and the remaining (18.52%) had capsular polysaccharide that were not typed by type 5 or type 8 antibodies. MRSA isolates were more likely to have different capsular polysaccharides types: CP5, CP8, and non 5 nor 8 capsular polysaccharides.

The antibiotic susceptibility testing revealed that the *S. aureus* strains fitted in 14 antibiogram types, with the type showing resistance only to Penicilin and Ampicillin prevalent in 48 out of 81 isolates (59.25%).

For better understanding of the immune response in ESRD patients to nasal *S. aureus*, we used enzyme-immuno linked assay to measure the concentration of anti-CP5 and anti-CP8 antibody. The heterogeneity of the antibody level among ESRD patients in both carrier and non-carrier emphasized that there is no correlation between nasal carriage of *S. aureus* and human immune response, and that high antibody concentration is not positively related to *S. aureus* nasal carriage in ESRD patients.

ألملخص

يعتبر حمل مرضى غسيل الكلى لبكتيريا المكوّرات العنقودية الذهبية في الأنف أحد العوامل التي تجعلهم أكثر عرضة لإصابة الأجهزة الداخلية بهذه البكتيريا عبر الدم.

خلال الفترة الزمنية من شهر أيار للعام 2004 إلى شهر كانون ثاني للعام 2005 تم عمل دراسة مسحة ل 99 حالة من مرضى الذين يعانون من فشل كلوي، في المستشفيات التالية: مستشفى رام الله ، مستشفى المطلع، و مستشفى عالية الحكومي لمراقبة نسبة حملهم لبكتيريا المكورات العنقودية الذهبية. ووجد أن 41 من مجموع ال99 ونسبتهم 41.4% كانوا حاملين لهذه البكتيريا. مع عمل الدراسة التتابعية لأنماط حملهم لهذه البكتيريا تم أخذ مسحات من الأنف بشكل دوري ، وقد تم تمييز وجود ثلاثة أنماط من أنماط حملهم لهذه البكتيريا: الحاملين الدائمين ونسبتهم 17.17% ، والحاملين لها بشكل متقطع ونسبتهم 24.24% والغير حاملين لها ونسبتهم 58.6%.

بعد عمل فحص النمط المصلي لمجموع البكتيريا العنقودية تبين أن معظم هذه البكتيريا المعزولة طبيا تحمل كبسولة متعددة السكريات بعدة أنماط مصلية على النحو التالي: 48.15% لديها كبسولة من متعدد السكريات النوع الخامس، 33.33% لديها كبسولة متعددة السكريات من النوع الثامن والنسبة المتبقية 18.52%لديها كبسولة متعددة السكريات ليست من النوع الخامس أو الثامن.

نتيجة لفحص البكتيريا المكورة العنقودية الذهبية والتي عندها مقاومة للميثيسيلين، تبين أن نوعها المصلي ليس مرتبطا بنوع معين من الكبسولة المتعددة السكريات حيث ممكن أن تكون من النوع الخامس أو ألثامن أو غيرهم.

بعد فحص البكتيريا العنقودية للمضادات الحيوية والتي عزلت من المرضى يمكن تصنيفها في 14 نمط للحساسية للمضادات الحيوية، مع وجود النمط السائد والذي أظهر مقاومة فقط للبنسيلين والأمبيسيلين ، وقد كانت نسبة هذا النمط 48 من 81 أي بنسبة 59.25%.

لدراسة العلاقة بين رد الفعل المناعي لدى المرضى الذين يعانون من الفشل الكلوي ووجود البكتيريا العنقودية في ألأنف، استخدمنا فحص الاليزا لقياس تركيز الأجسام المضادة للكبسولة المتعددة السكريات من نوع الخامس والثامن. و قد تبين أن عدم التجانس في مستوى ألأجسام المضادة أثبت عدم وجود علاقة إحصائية ايجابية بين حمل البكتيريا العنقودية في ألأنف وبين رد الفعل المناعي لديهم ، وأن وجود تركيز عالي من ألأجسام المضادة لديهم ليس له علاقة ايجابية مع حملهم لهذه البكتيريا في ألأنف.

Chapter One

Introduction

Staphylococcus aureus

Staphylococcus aureus is the most common cause of serious hospital-acquired infections, including blood stream infections and post-operative wound infections. The increased resistance of *S. aureus* to many different antibiotics is a growing source of concern in the medical community.

Staphylococcus aureus is a gram-positive facultative anaerobic, non-sporulating, non-motile, catalase positive, oxidase-negative and coagulase-positive cocci arranged in clusters resembling grapes. It is often β-hemolytic and forms large yellow colonies on blood agar and it grows well at a temperature range from 15˚C to 45˚C and at a salt (NaCl) concentration as high as 15%. Based on laboratory investigations *S. aureus* is distinguished from the other staphylococcal species on the basis of the gold pigmentation of colonies and positive results of coagulase, mannitol fermentation, and deoxyribonyclease tests (Lowy, 1998).

The cell wall of *S. aureu*s is a typical gram positive cell wall with thick and highly cross-linked peptidoglycan layer. The peptidoglycan consists of glycogen strands of N-acetyglucosamine and N-acetylmuranic acid disaccharides cross linked by tetra-peptides consisting of L-alanine, D-

glutanime, and D-alanine and L-lysine that are attached to the N-acetylmuramic acid (Geisbrecht er al., 1998). Another common feature in the cell wall is teichoic acid which consists of almost 50% of total mass of the cell wall. Teichoic acid functions in the specific adherence of *S. aureus* to mucosal surfaces (Elmer et al., 5th ed)

Staphylococcus aureus has a number of virulence factors that are coded with a complicated coordination between several regulatory systems. For the majority of diseases caused by *S. aureus*, it is difficult to determine precisely the role of each given factor. However these virulence factors are involved in adhesion, host defense evasion and tissue penetration. Such virulence factors of *S. aureus* include proteins, toxins and capsular polysaccharides. Fibrinogen-binding protein (FBP) and collagen binding protein are among *S. aureus* surface proteins, named as microbial-surface components recognizing adhesive matrix molecules (MSCRAMN) of human endothelial fibronectin (Lowy, 1998). The attachments of FBP to host endothelial fibronectin is considered necessary for efficient internalization of *S. aureus* into epithelial cells and phagocytes, even without the need for *S. aureus* specific receptors (Sinha et al., 2000). Protein A is another surface protein of *S. aureus* that has the ability to interfere with opsonization and ingestion of the bacteria by polymorphonuclear cells

(PMNs), as it binds to the Fc region of the immunoglobulin IgG molecules thus preventing opsonization (Elmer et al., 5th ed).

Staphylococcus aureus produces a variety of exoproteins or enzymes that contribute to *S. aureus* ability to cause disease by either degrading the host tissue to use it as nutrient needed for growth of the bacteria itself, or facilitate the penetration of *S. aureus* deeper into host tissue. Such enzymes include hemolysins (α, β, γ, and δ). α-hemolysin has a lysing effect on human PMN cells and red blood cells (RBC) by forming a pore in the cell membrane, thus allowing the efflux of Potassium (K+)and influx of Sodium (Na+) and Calcium (Ca++), causing osmotic swelling and rupture of the cells (Dinges et al., 2000). Fibrinolysins, Hyaluronidase and Lipase are also among *S. aureus* enzymes that allow and facilitate the spread of the bacteria to adjacent areas by the break down of fibrin clot and hydrolysis of intracellular matrix of mucopolysaccharides (Elmer et al., 5th ed)

Staphylococcus aureus produce a wide variety of toxins either exotoxins or endotoxins that aid in the pathogenesis of *S. aureus*. Toxic-shock syndrome-toxin 1(TSST-1) is one of the exotoxins of *S. aureus* that cause toxic-shock syndrome characterized by hypotension, hypoalbuminemia and edema. *Staphylococcal* food poisoning (SFP) is a form of gastroenteritis that results from the ingestion of one of the *S. aureus*

enterotoxins (SEs) on food contaminated with *S. aureus* (Dinges et al., 2000).

Capsular polysaccharide of *Staphylococcus aurus*

Bacterial capsular polysaccharides (CP) are carbohydrates polymers made up of a repeating saccharides units. Most *S. aureus* strains produce external capsules that were first described in 1931. Eleven antigenically distinct capsular polysaccharides are recognized (Karakawa et al., 1988). Among these eleven capsular serotypes capsular type 5 and 8 predominate among clinical isolates of *S. aureus* (Naw'as, 1998; M Roghmann, 2005). Any other *S. aureus* bacteria that do not react with antibodies to capsular type1, capsular type 2, capsular type 5 and capsular type 8 are called non-typeable (O'Riordan and Lee, 2004). The predominance of capsular type 5 or 8 among human isolates of *S. aureus* varies form one study to another (Essawi, 1998; Roghmann, 2005).

Based on biochemical characteristics of the capsular polysaccharides, both capsular type 5 and type 8 are very similar to each other according to their back bone structure, but serologically these two capsular polysaccharide are serotype specific (O'Riordan and Lee, 2004), and there is no immunological cross-reactivity between them as each of them can

produce specific antibodies that are effective in inducing opsonophagocytic killing of *S. aureus* by leukocytes. This is a proved evidence that capsular polysaccharides of *S. aureus* are associated with host immunity to the organism (Karakawa et al., 1988).

The expression of capsular polysaccharide type 5 and type 8 by *S. aureus* in vitro is affected by both environmental and bacterial culture conditions. *Staphylococcus aureus* grown on solid agar media, in glucose containing media –as carbon source under Oxygen supply – or under iron limitation, will produce higher concentration of capsular polysaccharide than bacteria grown in broth medium, or grown on artificial culture media (Thakker, 1998; Poutral,1995; Lee, 1993). Carbon dioxide (CO_2) acts as a down regulator for CP type 5 and CP type 8 expression, as growth of *S. aureus* with 5% CO_2 causes a decrease in both capsular polysaccharide 5 and capsular polysaccharide 8 expression (Herbert al., 2001).

Although capsular type 5 and type 8 *S. aureus* strains produce smaller amounts of capsules compared to type 1 and type 2 strains (O'Riordan and Lee, 2004), still capsular type 5 and type 8 play very important role in the pathogenesis of *S. aureus* as they specifically bind to human cells, including epithelial cells, endothelial cells and monocytes in a dose and calcium-dependent manner. As a result this interaction between *S*. a*ureus* capsular

polysaccharide and host cells is needed for initiation of *S. aureus* invasion of the cells (Soel et al., 1995). The role of capsular type 5 in *S. aureus* virulence has been proved in 1998 when the bacteria with high concentration of capsular type 5 were more virulent than mutant strains defective in capsular type 5 expression (Thakker et al., 1998). In 2002 it was also proved that over production of capsular type 8 caused the persistence of capsular polysaccharide positive bacteria in the blood stream, liver and the spleen of bacteremia infected experiment mouse (Luong and Lee, 2002).

The mechanism of antiphagocytic ability of *S. aureus* capsular type 5 and type 8 is based on its ability to inhibit complement mediated opsonization. Both capsular type 5 and type 8 mask or cover C3 fragments attached to the bacterial cell wall preventing it from binding to the complement receptors of the phagocytic cells (Cunnion et al., 2001). This was confirmed in the year 2003 when the presence of anti-capsular antibodies during complement activation improved the recognition of C3 fragments bound to positive *S. aureus* by complement receptors even when the number of C3 molecules is not that high (Cunnion et al 2003).

Methicillin Resistant *Staphylococcus aureus*

Methicillin – resistant *S. aureus* (MRSA) is an important pathogen, and is a major cause of nosocomial infections. Methicillin resistant *S. aureus* is common in many areas of the world, with different prevalence rates from one geographic region to another, or even from one section to another of one hospital (Fluit et al., 2001). Methicillin resistant *S. aureus* is primarily mediated by the *mecA* gene that encodes for penicillin- binding protein 2a (PBP2a), which reduces the binding affinity for all beta lactam antibiotics (Warren et al., 2004). There is no *mecA* homolog in susceptible strains. Both susceptible and resistant strains of *S. aureus* produce four major PBPs: PBPs1, 2, 3, and 4. Penicillin-binding proteins 1, 2, and 3 that have high affinity for most β-lactam antibiotics are essential for cell growth and for the survival of susceptible strains, and binding of β-lactams by these PBPs is lethal. In methicillin-resistant cells, PBPs 2a with its low affinity for binding β-lactam antibiotics, can substitute for the essential functions of high-affinity PBPs at concentrations of antibiotics that are otherwise lethal (Chambers, 1997). Nasal colonization of MRSA has been shown to be a risk factor for nosocomial infections (Kluytmans, 1997; Watanable, 2000), as well as community-acquired infections. In recent years, community acquired MRSA infections have been emerging as a world wide problem (Akram et al.,

1998), that could be transmitted from the hospital to the community through discharged patients, or even health care workers (Liang, 2005; Charlebois, 2002). Most cases of community-acquired MRSA infections occur in patients with underlying medical conditions and are in frequent contact with the health care system, such as hemodialysis patients (Salgado et al., 2003). The increase of MRSA infections – nosocomial or community acquired--created great obstacles for medical professionals, due to failure of treatment of the resistant *S. aureus*. As a result infections caused by MRSA are usually treated with Vancomycin. Vancomycin is toxic and the emergence of Vancomycin-resistant strains of *S. aureus* first in Japan in 1996 and in the united states, caused great concern that such resistance could result in high mortality and morbidity rates (Smith et al., 1999)

Carriage of *Staphylococcus aureus*

Humans are natural reservoir of *S. aureus* that colonizes skin and mucus membranes of healthy people. Usually *S. aureus* colonizes the moist squamous epithelial cells of the human nares (Cole et al., 2001). *Staphylococcus aureus* nasal carriage has been studied extensively in both patients and healthy individuals (Kluytmans, 1997; Cole, 2001; VandenBergh, 1999; Chapoutot, 1999; Ahmed, 1998; Luzer, 1990). The

nasal carriage of *S. aureus* in healthy adult population has been described to fall into three patterns: persistent carriage approximately 20%, intermittent carriage 60%, and non carriage 20% (Kluytmans et al., 1997). The criteria used to identify these carriage patterns have varied from one study to another depending on the number of nasal specimen cultures that are done, the follow-up period, and the interpretation of data (VandenBergh et al., 1999) The typing of *S. aureus* strains isolated from individuals identified as persistent carrier showed that almost all stable carrier were persistently infected with the same strain of *S. aureus* and that changes in the strain by time seldom occurred (Hu et al., 1995).

Age of the studied population affects the proportion of nasal carriage, as infant carriage rate ranges from 50% during the first eight weeks of life to 21% by six months of age (Peacock et al., 2003). Very young school students have higher nasal rate of *S. aureus* than older individuals (Liang et al., 2005). In addition the health condition of people also affect the percentage of carriage of *S. aureus*. The percent of carriage rate in hospitalized , insulin-dependent diabetes, HIV-positive patients, and also in those on hemodialysis or peritoneal dialysis is higher than that in healthy individuals (Kluytmans, 1997; Chapoutot, 1999; Ahmed, 1998).

The ability of *S. aureus* to adhere to human endothelial cells is

affected by both human and bacterial factors. The binding of *S. aureus* to cell-associated mucus is greater than to non mucus coated epithelial cells. This binding between *S. aureus* and human nasal mucus occurs by specific adhesion-receptor interactions involving the fibronectin bridging between *S. aureus* fibronectin-binding proteins (FnBPs) and human cell fibronectin receptors that proved to be sufficient for invasion of *S. aureus* to host cells (Shuter, 1996; Sinha, 2000).

Human nasal fluid also contributes to the adhesive process of *S. aureus*. Although the nasal fluid from healthy carriers contains both high concentration of neutrophil derived peptides that indicates a neutrophil-mediated host response to *S. aureus* colonization, and also high concentration of anti-microbial peptides secreted in the nose of carriers compared to non-carriers, but still the nasal fluid from carriers is defective in killing the self nasal isolates of *S. aureus*, consequently involved in affecting the nasal carriage rate (Cole et al., 2001).

The anti-microbial activity of nasal fluid of human includes both immunoglobulin A (IgA) and immunoglobulin G (IgG). The reduced concentration of these anti-microbial substances due to the abnormalities of nasal fluid movements across epithelial, results in reduction of the anti-microbial activity of the nasal secretions, (Cole et al., 1999). Moreover

epithelial cilia continuously transport mucus with particulate matters and microbes down the oro-pharynx, where they are swallowed and may result in reduction in bacterial concentration. Still in some cases of people having thick mucus or ciliary dysmotility a delay in the clearance of the bacteria from the airway occurs and may permit the microbes to adapt to the damaging effects of secretions and to recover and resume growth (Cole et al., 1999). Capsular polysaccharides expression of *S. aureus* is negatively correlated to the adhesion of the bacteria to the endothelial cells, as the induction of CP type 5 results in reduced adhesion of *S. aurues* (Pohlmann et al., 2000).

Nasal *S. aureus* has been identified as a risk factor for hospital-acquired, as well as community acquired infections. Hospital acquired infections are mostly caused by the patient's own nasal *S. aureus* in surgical patients (Ahmed, 1998; Perl, 2002), intensive care unit patients (Eiff, 2001; Singh, 2003), patients with peritoneal dialysis (Sewell, 1982; Luzer, 1990), hemodialysis patients (Chow et al., 1989), and broncho-pulmonary infected patients (Watanabe et al., 2000).

The reservoir of *S. aureus* in hospital infections is not clear, as some patients are already colonized with *S. aureus* at the time of their hospital admission, where others become colonized during their stay in the hospital.

Health care workers are assumed to be one of those sources of *S. aureus* transmission to the patients. Medical staff members who are colonized with *S. aureus* (VandenBergh, 1999; Ahmed, 1998; Cespedes, 2002), or have direct contact with colonized patients, can contaminate their hands and transmit the bacteria to other patients during their daily routine care of the patients in the hospitals or other health care centers (Eveillard, 2004; Lee, 2000; Pittet, 1999).

End stage renal disease patients are at risk of acquiring *S. aureus* infections from the hospitals. End Stage Renal Disease (ESRD) is a slow, progressive loss of the kidney ultra-filtration capacity, that results in the accumulation of metabolic waste products, disturbance of kidney's endocrine functions and electrolytes balance. Renal failure could be acute or chronic: Acute renal failure is defined as a rapid deterioration in renal function, sufficient to result in accumulation of nitrogenous wastes in the body. In acute renal failure the kidney has a remarkable capacity to regain function following the various forms of acute renal failure. Chronic renal failure is a more severe renal injury that is not reversible and leads to progressive destruction of nephron mass. The most common causes of this irreversible failure are: Glomerulonephritis, tubulointerstitial diseases, diabetic nephropathy, and nephrosclerosis.

End Stage Renal Disease is irreversible and fatal if not early detected and properly treated through dialysis or transplantation. Kidney transplantation is severely limited due to the shortage of suitable donors, the incidence of organ transplant rejection, the age, and health of many ESRD patients. The majority of patients, therefore, must rely on dialysis for the remainder of their lives.

Dialysis procedures are based on circulating blood on one side of a semi-permeable membrane against a physiologic electrolyte solution on the other side to remove the accumulated by products. Hemodialysis uses an artificial membrane, while peritoneal dialysis uses the peritoneal membrane of the abdomen, which is a large membrane rich in blood vessels, to act as a filter to eliminate waste products and excess fluid from the blood. Both hemodialysis and peritoneal dialysis are the two prevailing methods of dialysis.

Impaired immunity in uremic patients makes them more susceptible to infectious processes. Infection can have a major impact on patients with end-stage renal disease. Both typical and opportunistic organisms can infect patients with end-stage renal disease (Chi et al., 2004). Because of the need for connection between the outside of the body and the blood stream of the patient during dialysis sessions, both hemodialysis and peritoneal dialysis

techniques predispose the patients to such infections. As a result both forms of dialysis require strict aseptic technique to reduce such infections, especially infections related to non-hematogenous routes that occur by direct inoculation of the access site. An effective hand-washing system by medical staff using an antimicrobial agent is superior to ordinary hand washing, and reduces the rate of nosocomial infections (Doebbeling, 1992; Pittet, 1999). Furthermore the application of Mupirocin three times a week to hemodialysis catheter exit sites is associated with a marked reduction in line-related sepsis and prolongation of catheter survival (Johnson et al., 2002).

In hemodialysis patients, *Staphylococcus aurues* constitutes one of the major pathogens that is responsible for high morbidity and mortality rate. It is the most frequently isolated pathogen at the vascular access site infections associated with bacteremia and septicemia (Saxena, 2003; Kluytmans, 1997). In peritoneal dialysis patients, *S. aureus* also causes exit site infections and peritonitis, both are difficult to treat and may require alteration of dialysis therapy (Luzer et al., 1990).

The carriage rate of *S. aureus* in ESRD patients is higher than that of healthy individuals or even non-ESRD patients (Kluytmans et al., 1997). It is not clearly understood why the ESRD patients have higher carriage rates of *S. aurues* than others. Nasal carriage of *S. aureus* is considered an

important risk factor for ESRD patients treated by hemodialysis and peritoneal dialysis. ESRD patients are prone to *S. aureus* infections more frequently than non carrier (Swell, 1982; Chow and Yu, 1989; 1986), due to their decreased immunity, the use of prosthetic devices and multiple needle puncture that not only break the normal host defense and give direct access to sterile body sites, it also provides a site for colonization (Peacock, 2002; Saxena, 2003). End Stage Renal Disease patients with persistent *S. aureus* nasal carriage tends to have colonization with a single phage type (Yu et al., 1986). In many studies the strains from the nares and the strains causing the infection are similar in phage typing and antibiotic profile (Luzar, 1990; Swell,1982; Chow and Lu, 1989).

As a preventive measurements nasal *S. aureus* can be eliminated using intranasal Mupirocin (Perl 2002; Johnson, 2002), or by the use of topical antibiotics Polysporin ointment applied to the central venous catheter insertion site for hemodialysis patients in whom permanent vascular access can not be achieved (Lok et al., 2003).

Recently vaccination with *S. aureus* conjugate vaccine (StaphVax) to patients receiving hemodialysis proved to result in partial immunity against *S. aureus* infections for approximately 40 weeks (Shinefield et al., 2002). *Staphylococcus aureus* capsular type 5 and type 8 capsular polysaccharides

conjugate vaccines were developed and evaluated for their safety and immunogenicity in healthy volunteers (Fattom et al., 1993). Capsular polysaccharides of *S. aureus* alone are poor immunogens, therefore their immunogenicity is enhanced by conjugation with recombinant exoprotein A that is a nontoxic variant of *Pseudomonas auroginosa* (Shinefield et al., 2002). The vaccine was well-tolerated by immunized volunteers and neither the conjugates nor the capsular polysaccharides caused significant adverse reactions. The antibodies produced as a result of this conjugate vaccine were found to be mainly of IgG class immunoglobulin, and were found to mediate in vitro opsonophagocytosis of the type-specific *S. aureus* by human poly-morphonuclear cells (Fattom et al., 1993).

AIMS OF THE STUDY

End Stage Renal Disease patients who are *S. aureus* nasal carriers, are at continuous risk of developing infections. Screening ESRD patients periodically for nasal carriage of *S. aureus* and treatment of only those who are colonized will lead to better prevention of serious *S. aureus* infections. The aim of this study is to obtain more information on issues related to *S. aureus* nasal carriage in ESRD patients.

The specific aims of this study were to:

1 Screen for *S. aureus* nasal carriage in ESRD patients.

2 Characterize S. *aureus* nasal isolates based on capsular polysaccharides serotypes.

3 Study the role of natural carriage of *S. aureus* or previous infections with *S. aureus* on anti-CP type 5 and anti-CP type 8 concentrations in ESRD patients.

Chapter Two

Materials and Methods

Design and data collection

The present study was carried out between May 2004 to January 2005. The study population consists of three different groups: ESRD patients, Non-ESRD patients, and the medical staff. The number of ESRD patients enrolled in the study was 99 from three different hospitals: 34 from Ramallah Governmental Hospital in Ramallah, 48 from Augusta Victoria Hospital (AVH) in Jerusalem, and 17 from Alia Governmental Hospital in Hebron. The 25 NESRD patients were from Ramallah hospital. The 18 medical staff were from the three mentioned hospitals: five from Ramallah hospital, five from AVH, and eight from Alia hospital.

* Verbal consent was obtained from the patients in the presence of authorized physician following explanation about the study and its objectives, and also authorized access to patients medical records was obtained.

Collection and processing of the samples

Carriage of *S. aureus* was determined by obtaining nasal swab specimens from both anterior nares using sterile dry cotton-wool swabs (LAB service S.P.A). The first two swabs were taken at an interval of one week apart, the other two swabs were taken at subsequent dialysis sessions within about three months . The blood samples were taken on the same day with the first nasal swab. Serum samples were then stored at -80˚C for further analysis.

Microbiological methods

Nasal swabs were inoculated on both 5% sheep blood agar and mannitol salt agar MSA (Oxoid). The plates were incubated for 24 hours at 35˚C and observed for the growth of suspected *S. aureus* colonies. *Staphylococcus aureus* isolates were identified on the basis of colony morphology golden pigmentation of colonies, β-hemolytic activity on blood agar, fermentation of mannitol, Gram staining, bound slide coagulase test,

tube coagulase test (BD BBL), and Deoxyribonuclease (DNase) test. *Staphylococcus aureus* isolates were suspended in 1:1 glycerol and thioglycollate broth and stored at -80˚C for further analysis.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of all isolates of *S. aureus* to different antimicrobial agents was determined by the disk agar diffusion method in accordance with the National Committee for Clinical Laboratory Standards (NCCLS 1993). The following antimicrobial disks and concentrations used were: Penicillin (10 U/ml), Oxacillin (1µg/ml), Ampicillin (10µg/ml), Tetracycline (30µg/ml), Clindamycin (2µg/ml), Vancomycin (30µg/ml), Erythromycin (15µg/ml), and Cefuroxime 30uµg/ml). The results were recorded after 18-24 hours of incubation at 35˚C. A standard strain of *S. aureus* (ATCC 25923) was used as a control.

Capsular polysaccharides serotyping

Staphylococcus aureus isolates recovered from the study patients (ESRD patients) and the controls (NESRD patients and the medical staff), were streaked on blood agar and incubated overnight at 37˚C. The next day, a single colony was streaked on modified Columbia agar (Difo laboratories,

Detroit, Michigan) supplemented with 0.5% $MgCl_2$ and 1.5% $CaCl_2$ and incubated for 24 hours at 37˚C. Several colonies grown on modified Colombia agar were suspended in 500 µl of 0.9% sterile saline and tested by slide agglutination with rabbit polyclonal antibodies specific to capsular polysaccharides type 5 and type 8 provided and standardized by Nabi (Rockville, Maryland). Positive agglutination was observed by visible clumps within 30 seconds using clean microscopic slide.

Determination of anti- CP type 5 and anti- CP type 8 in the sera of the ESRD patients.

Anti-CP type 5 and anti-CP type 8 titers were determined by an enzyme-linked immunosorbent assay (ELISA) as follows. Microtiter plates were coated with 100 µl of 1µg/ml of either CP5 or CP8 in PBS , and the plates were incubated at room temperature overnight. To eliminate non-specific adsorption, the antigen (CP5 or CP8) was blocked by adding 200µl of 1% bovine serum albumin (BSA) in PBS to each well for one hour at room temperature. The wells were then washed 3 times with 300µl of PBS containing 0.1% Brij. The patients sera were diluted 1: 300 with PBS-Brij. Then 200µl of the diluted sera were added to the appropriate wells; each serum sample was two-fold serially diluted down the plate in parallel with

reference antibody (anti-CP5 and anti-CP8) and with negative control. The reference antibody (anti-CP5and anti-CP8) was pre-diluted 1: 4000. The plates were incubated for one hour at 37˚C. The wells were then washed 3 times with 300µl PBS-Brij. Peroxidase-conjucated goat anti-human IgG antibody (lot 59663 & lot 65774 – Kirkegaard and Perry Laboratories) was diluted in PBS-Brij to 1:10:000. One hundred microliter of the anti-human antibody were added to each well. Plates were then incubated at 37˚Cfor one hour and then washed 3 times with 300µl of PBS-Brij. One hundred microliters of freshly prepared peroxidaze substrate system (Kirkegaard and Perry Laboratories- KPL) prepared as 1:1 mixture of Hydrogen peroxide (H_2O_2) and Tetra methyl benzoate (TMB) were added to each well and the plate was incubated for 10 minutes at room temperature. The enzyme-substrate reaction was stopped by adding 100µl of 1M Phosphoric acid to each well. Finally the absorbance was measured at 450nm using ELISA reader, and the result for each serum sample was expressed as concentration µg/ml.

Calculation of antibody concentration

1- The linear range of both the reference antibody and serum samples was defined by visual inspection of the data.

2- The cut off value or the minimum optical density (OD) to take into account was defined as the mean optical density of the negative control plus two standard deviation.

3- Total optical density (TOD) was calculated for the reference anti-CP and for each serum by multiplying each dilution factor with its OD, then the TOD was averaged for the reference anti-CP and each serum samples.

The concentration of anti-CP in each serum sample was calculated using the formula: $\frac{\text{Serum sample TOD}}{\text{Reference TOD}} \times \text{Reference concentration} = \mu g/ml$

Statistical Analysis:

Descriptive statistics were used to summarize the data. The t-test was used for statistical comparisons between anti-CP type 5, anti-CP type 8 antibody concentration and nasal carriage status in ESRD patients, also between nasal carriage and sex, age, and Non-communicable disease state of the patients.

Chapter Three

Results

Screening for *S. aureus* carriage in ESRD patients and in NESRD patients:

In total, the ESRD patients enrolled in the study were 99 patients, males 50.5% and females 49.5%. The age range was from 4-79 years old with a median of 50 years for males and 48 years for females. A total of 55 patients had either Diabetes mellitus or Hypertension or both. Five (5.1%) patients had Diabetes mellitus and 36 (36.4%) had Hypertension while 14 (14.1%) were having both Diabetes mellitus and Hypertension, and 44 (44.4%) had neither DM nor HTN. The twenty five NESRD patients enrolled in the study were 13 (52%) males, 12 (48%) females, 4 (16%) diabetes mellitus, 8 (32%) Hypertension, 6 (24%) with both Diabetes mellitus and Hypertension,, and 7 (28%) had neither DM nor HTN. According to the vascular access type 88 patients had a fistula, 2 had graft, and 9 had subclavian as an access. During the study period 18 of the 99 ESRD patients died (table 1).

Of the 99 patient's nasal swabs 41 (41.4%) were positive for *S. aureus* and 58 (58.6%) were negative. For the NESRD patients 3 (12%) were

positive for *S. aureus* and 22 (88%) were negative for nasal *S. aureus* (Table 2). Seventeen patients (17.17%) of the ESRD patients were persistent carrier, 24 patients (24.24%) were intermittent carriers and 58 patients (58.6%) were non- *S. aureus* carrier (Table 3). The carrier index for each patient was defined as the number of nasal swab that is positive for *S. aureus* divided by the total number of nasal swab specimen cultures performed for that patient. Persistent nasal carrier are those patients with carrier indices of 0.7 or higher, intermittent carrier are those with carrier indices between 0.1 and less than 0.7, and non-carriers are those with carrier indices of zero (VandenBergh et al., 1999)

Table 1. ***General characteristics of ESRD***

	Male	**female**	**Graft**	**Fistula**	**Subcla-vian**	**DM**	**HTN**	**DM+ HTN**
Ramallah	**20**	**14**	**1**	**33**	**0**	**2**	**13**	**2**
AVH	**20**	**28**	**0**	**39**	**9**	**2**	**22**	**11**
Alia	**10**	**7**	**1**	**16**	**0**	**1**	**1**	**1**
Total	**50**	**49**	**2**	**88**	**9**	**5**	**36**	**14**

ESRD: End Stage Renal Disease Patients

Table 2: ***Description statistics of nasal carriage of S. aureus in ESRD and NESRD patients.***

	ESRD		NESRD	
	Number	**Percent**	**Number**	**Percent**
Carrier	**41**	**41.4%**	**3**	**12%**
Non-carrier	**58**	**58.6%**	**22**	**88%**
Total	**99**	**100%**	**25**	**100%**

ESRD: End Stage Renal Patients

NESRD: Non-End Stage Renal Patients

Table 3*: Descriptive statistics for the nasal carriage pattern of S. aureus In ESRD Patients*

	Frequency	Percent
Persistent (P)	**17**	**17.17 %**
Intermittent (I)	**24**	**24.24%**
Non- Carrier (NC)	**58**	**58.6%**
Total	**99**	**100%**

P: Carriage index ≥ 0.70

I: Carriage index $0.1 \leq x < 0.70$

NC: Carriage index = 0

Our data showed that there is no significant difference in the percent of nasal carriage between the different age-groups. The percent of nasal carriage in the age-group ≤ 20 was 45.5%, in the age-group 21-40 was 46.2%, in the age-group 41-60 was 39.5%, and in the elderly group ≥ 60 was 38.5% (Table 4). According to the sex also there was no significant difference between males and females, with males 48% and females 34.7% (Table 5).

Screening for *S. aureus* nasal carriage in the medical staff showed that of the 18 medical staff 5 (27.8%) were *S. aureus* positive nasal carrier.

The percent of *S. aureus* nasal carriage in the ESRD patients showed no significant difference between those patients that have non-communicable disease and those who do not have. NCDs patients had a percent of nasal carriage of 41.8%, while the Non NCDs patients had a percent of nasal carriage of 40.9% (Table 6).

Among the eighty one of nasal *S. aureus* isolates , the predominant capsular type was type 5 with a prevalence of 48.15% (39/81) while the prevalence of capsular type 8 was 33.33% (27/81). The remaining 15 isolates (18.52%) were non-typeabl. Among the persistent carriers the prevalence of both capsular type 5 and type 8 was 58.8% (10/17), and 35.3% (6/17) respectively, and 5.9% (1/17) were non typeable.

Table 4*: Descriptive statistics of S. aurens nasal carriage in different age groups in ESRD patients.*

	≤20		21- 40		41-60		≥60	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Carrier	10	45.5%	6	46.2%	15	39.5%	10	38.5%
Non-carrier	12	54.5%	7	53.8%	23	60.5%	16	61.5%
Total	22	100%	13	100%	38	100%	26	100%

Table 5: ***Descriptive statistics of S. aureus nasal carriage according to gender of ESRD patients.***

	Male		**Female**	
	Number	**Percent**	**Number**	**Percent**
Carrier	24	48%	17	34.7%
Non- carrier	26	52%	32	65.3%
Total	50	100%	49	100%

Table 6: ***Descriptive statistics of S. aureus nasal carriage according to Non-Communicable disease state of ESRD patients.***

	NCD		Non NCD(s)	
	Number	**Percent**	**Number**	**Percent**
Carrier	23	41.8%	18	40.9%
Non- carrier	32	58.2%	26	59.1%
Total	55	100%	44	100%

NCD(s) : Non- communicable disease

Capsular typing of the *S. aureus* isolates recovered from the NESRD patients were 33.3% (1/3) have CP type 8 while 66.7% (2/3) did not express neither type 5 nor type 8 capsule. Most of the isolates from the medical staff 60% (3/5) have CP type 5 and 40% (2/5) have CP type 8.

The antimicrobial susceptibility of the 81 nasal isolates was tested by disc-agar diffusion. Table 7 shows the antibiogram types where the majority of the nasal isolates (48/81; 59.3%) were of antibiogram type A, resistant only to Penicillin and Ampicillin. Table 8 lists the distribution of nasal *S. aureus* isolates by capsular and antibiogram types. The biggest clusters of strains is the one showing the capsular type 5 and antibiogram type A constituting (27/81; 33.3%).

The sera obtained from the 99 ESRD patients showed a great variability in the levels of both anti-CP5 and anti-CP8 ranging from 1-137.6 µg/ml, and 3.1-94.2 µg/ml respectively. Their was no significant difference in the antibody level when the anti-CP5 and anti-CP8 antibody concentration was tested against the nasal carriage status of the patients. Also there was no relation between anti-CP5 and anti-CP8 antibody concentration and *S. aureus* type 5 and type 8 CP carriage. By comparing the concentration of our patients antibody to that of a previous study done in 1993 on pre-immunized patients, there was a similarity in anti-CP5, CP8 and antibody concentration.

Table 7*: Nasal Staphylococcus aureus antibiogram types identified from The ESRD patients*

type	P	Am	Ox	Va	Cc	E	Tet	Cxm
A	r	r	s	s	s	s	s	s
B	r	r	s	s	s	s	r	s
C	r	r	r	s	s	s	s	s
D	s	s	s	s	s	s	s	s
E	r	r	s	s	r	s	s	s
F	r	r	r	s	s	s	r	s
G	r	r	s	s	s	r	s	s
H	r	r	r	s	s	s	s	r
I	r	s	s	s	s	s	s	s
J	r	r	s	s	r	r	s	s
K	r	r	s	s	s	r	s	r
L	r	r	r	s	r	r	s	r
M	r	r	r	s	s	r	s	s
N	r	r	s	s	s	s	s	r

Abbreviations: P, Penicillin ; Am, Ampicillin; Ox, Oxacillin; Va, Vancomycin; Cc, Clindamycin; E, Erythromycin; Tet, Tetracycline; Cxm, Cefuroxime; s, susceptible; r, resistant.

Table 8: *Nasal Staphylococcus aureus isolates distributed by capsular and antibiogram type*

Capsular type	Antibiogram types														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	Total
5	27	4	0	1	1	0	1	2	1	1	0	0	0	1	39
8	12	6	0	2	0	1	4	0	0	0	1	0	1	0	27
N	9	1	1	1	0	0	0	0	0	1	0	1	0	1	15
Total	48	11	1	4	1	1	5	2	1	2	1	1	1	2	81

Discussion

The present study was designed to determine the prevalence of nasal *S. aureus* among (ESRD, NESRD patients, health workers), characterize the nasal *S. aureus* isolates by capsular typing, and evaluate whether nasal carriage of *S. aureus* has an effect on the human immune response.

Staphylococcus aureus nasal carriage has been extensively studied in patients and in healthy individuals (Kluytmans, 1997; Cespedes, 2002; Saxena, 2003). We present here the first study of the prevalence of nasal colonization with *S. aureus* in ESRD patients done in the West Bank of Palestine. The result of this study showing that 41.4% of our ESRD patients are nasal carriers of *S. aureus* fit in well with the results obtained from the previous surveys that had evaluated the prevalence of nasal carriage of *S. aureus* in hemodialysis patients range between 30.1%-84.4% (Kluytmans et al., 1997). Although these results are in accordance with previous studies, still 41.4% in the developing countries is considered high, and efforts should be made to reduce this rate.

In the present study we identified 17.17% persistent carrier, 24.24% intermittent carrier, and 58.6% non carrier over the study period. The carriage pattern in our study is considered less than that established in other

studies of healthy individuals as 20% of individuals carry *S. aureus* persistently, 60% carry *S. aureus* intermittently, and 20% are persistently free of *S. aureus* (non-carrier) (Kluytmans, 1997; VandenBergh, 1999). The difference in the reported carriage pattern is postulated to be affected by certain obstacles concerning sample collection: as many of our patients died, some refused to give one or two of the nasal swabs, others used to take antibiotics at the time of sample collection, and few who were lucky to have kidney transplant and were excluded from the study. Furthermore in this study the 3 months interval between nasal swabs collection may had an effect on the nasal carriage pattern of the ESRD patients. Taking of antibiotics by the patients may affected the results, since the consumption of antibiotics whether oral or topical antibiotics do not eliminate nasal *S. aureus* completely, as a relapse rate of *S. aureus* nasal carriage occurs after the use of such antibiotics (Kluytmans et al., 1997).

In this study, male hemodialysis patients has a higher prevalence of nasal *S. aureus* carriage than did female hemodialysis patients (48% Vs 36.7%). Although there was no statistical significance in nasal carriage rate between males and females, a trend can be seen toward higher carriage rate in males that may play a role in future exposure to infections.

Furthermore the elevated *S. aureus* nasal carriage rate in ESRD patients of the age group ≤ 20 with 50% positive nasal carriage might be related to the close and direct bodily contact between these patients in particular those under the age of 10, that facilitate the transmission of *S. aureus* from one patient to another. Accordingly positive *S. aureus* nasal carrier may be considered a reservoir for transmission of *S. aureus* to the surrounding environment and to other people in direct contact with them. In addition other risk factors that might affect nasal carriage of *S. aureus* on our target population could be related to the immuno compromised health state of the patients, malnutrition, and the multi- punctures of their body.

Polysaccharide capsules are prevalent among *S. aureus* isolates from both commensally and pathogenic sources. Many studies have used the capsule polysaccharide typing scheme for typing infectious *S. aureus* in human (Essawi, 1998; Rogman, 2005). For the first time in the West bank of Palestine, the capsular polysaccharides typing scheme is used to study the distribution of CP types among nasal *S. aureus* isolates from ESRD patients. The majority of *S. aureus* nasal isolates (80.2%) were as expected and comparable with other studies, capsulated by either type 5 or type 8 capsule, with prevalence of type 5 (48.2%), (33.33%) of type 8, and (18.52%) non 5 nor 8 type.

In our country where antibiotics are freely dispensed over the counter, and hospitals lack policies regarding the use of these antibiotics, so using capsular typing in conjunction with antibiogram typing could be used as a simple, cheap strategy for control of *S. aureus* infections.

Human humoral immune response to *S. aureus* infections results in a variable antibody response against *S. aureus* cell wall antigens including Peptidoglycan (PG), Lipoteichoic acid (LTA), Fibrinogen Binding Protein (FBP), and Capsular polysaccharides (CP) (Dryla, 2005; Colque, 2000). The anti-staphylococcus antibody produced due to infections do not seem to be protective even in healthy people, and even the higher antibody concentration in the convalescent state of infection are only transient and decrease in level later post infection. Moreover the antibody levels against *S. aureus* do not differentiate significantly between healthy and diseased people as both populations showed to have varying levels of anti-staphylococcal antibody that do not correlate with health status of the people (Dryla et al., 2005). By comparing antibody concentration in our study with that of Dr Fattom study done in 1993, there was a similarity in antibody concentration of our patients with that of the pre-immunized patients of Dr Fattom (Fattom et al., 1993)

In this study the recognition of *S. aureus* by the immune system as measured by both anti-CP type 5 and anti-CP type 8 showed that ESRD patients have varying concentrations of circulating anti-CP5 and anti-CP8 antibodies. The poor correlation between *S. aureus* nasal carriage and anti-CP antibody levels indicates that this heterogeneity in the antibody level in the ESRD patients is independent of *S. aureus* nasal carriage. The variation in the antibody concentrations may have resulted from previous *Staphylococcal* infections, or non-specific antibody response that caused the variability in antibody level in both carrier and non-carrier ESRD patients.

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