UNIVERSITY OF CALGARY

Influence of *in vitro* bacterial urokinase responsiveness on the *in vivo* pathogenesis of methicillin-sensitive *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* in mouse models.

by

Shawna J. Yanke

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

DECEMBER, 1999

ABSTRACT

Previous studies reported that clinical isolates of *S. aureus* may respond differently following addition of UK to *in vitro* growth medium, suggesting that the plasminogen activator influenced bacterial growth (Hart et al., 1996 and Hart and Woods, 1994). Two animal models were developed to further investigate these findings in an *in vivo* setting. CD1 mice and uPA-/- mice were intravenously inoculated with *S. aureus* and found to develop bacterial colonization primarily of the kidney and some joints. The uPA-/- mice were more susceptible to infection than the CD1 mice and also showed bacterial colonization of the heart. Preliminary investigations revealed that an inoculation dose of 10^7 CFU per mouse was optimal with regard to the development of chronic infection.

Using these two mouse models, UK responsive and UK non-responsive *S. aureus* isolates were tested for *in vivo* correspondence to *in vitro* findings. From the results, it appears that the *in vitro* UK phenotype does not overtly influence *in vivo* pathogenicity. When bacterial colonization of the kidney and mortality *in vivo* were assessed, UK responsive and UK non-responsive *S. aureus* isolates displayed similar results. The MSSA isolates tested did however show increased virulence compared to the MRSA isolates tested. This investigation also indicated that gender has an influence on bacterial colonization. Female mice were more susceptible to MRSA colonization and mortality than identically treated male mice, and castration of CD1 male mice resulted in an increase in bacterial colonization of the kidney, knee joint and elbow joint.

ACKNOWLEDGEMENTS

First I would like to thank my supervisor, Dr. D.A. Hart, for his incredible guidance throughout my studies. I also extend my gratitude to the other members of my supervisory committee, Dr. D. Davies and Dr. M.E. Olson, for their suggestions and advice. Also, thank you to Dr. A. Buret for serving on my defense committee.

Next, I would like to acknowledge the expert histological assistance of Liz Middlemis. Her time and help are appreciated.

I am grateful to my parents, Lloyd and Barbara Yanke, who have supported me in every possible aspect through my many years of schooling. Their continuous encouragement and love have made my endeavors possible. I thank you Mom and Dad.

At last, I wish to thank my husband-to-be, Curtis. He has helped me through those trying times always providing me with positive words. His love and understanding have proved to be priceless. Thank you.

In memory of

Carter Hayden Yanke.

He will always be in my heart.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

CFU ..... colony forming units

DNA ..... deoxyribonucleic acid

GISA ..... glycopeptide-intermediate *Staphylococcus aureus*

H&E ..... hematoxylin and eosin

ID_{50} ..... infectious dose of 50 percent

MHV ..... murine hepatitis virus

MMA ..... methyl methylacrylate

mRNA ..... messenger ribonucleic acid

MRSA ..... methicillin-resistant *Staphylococcus aureus*

MSSA ..... methicillin-sensitive *Staphylococcus aureus*

PA ..... plasminogen activator

PBP ..... penicillin binding protein

PBS ..... phosphate buffered saline

PMNs ..... polymorphonuclear cells

SD ..... standard deviation

TMTC ..... too many to count

tPA ..... tissue-type plasminogen activator

TSST-1 ..... toxic shock syndrome toxin 1

UK ..... urokinase

uPA ..... urokinase plasminogen activator

CHAPTER 1

INTRODUCTION

1.1. *STAPHYLOCOCCUS AUREUS*

Staphylococcus aureus is one of the most important and widespread hospital pathogens known. It is a gram-positive coccus, often found as part of the normal flora of humans, which seizes the opportunity to cause disease in situations of host compromise.

1.1.1. Pathogenicity

How a microorganism causes disease is multifactorial, usually involving at least four different steps. The first step is adherence, which is necessary so that the bacteria can begin to colonize a specific area. Adherence is facilitated through attachment by bacterial adhesins (Salyers and Whitt, 1994). Staphylococci can possess over 8 different virulence factors involved in attachment (Projan and Novick, 1997). Multiplication of the microorganism follows, allowing bacterial colonization. To ensure survival, the bacteria now require means to evade host defense mechanisms. A number of the virulence factors that *Staphylococcus aureus* can produce to protect itself from host destruction are discussed below.

Some strains of *S. aureus* possess an extracellular polysaccharide capsule which covers the surface of the bacterium. The role of the capsule is believed to protect the bacteria from the inflammatory response of the host. Strains of *S. aureus* can be mucoid and encapsulated or non-mucoid and encapsulated. The latter are referred to as

"microcapsules" and are the most commonly found phenotype of clinical isolates (Wilkinson, 1983). Lee and colleagues have found that mucoid capsulated forms of *S. aureus* are more virulent than capsule-negative or capsule-deficient mutants, but microcapsulated strains are not more virulent (Lee et al., 1987; Albus et al., 1991).

Bacteria such as *S. aureus* can also coat themselves with host-like proteins to evade the host's immune response. Protein A of *S. aureus* can bind to the Fc portion of host antibodies, thus coating the bacteria with antibodies, but in a way that does not lead to opsonization of the bacteria (Salyers and Whitt, 1994).

Other factors such as lipase, leukocidin, staphylokinase and fatty acid modifying enzyme can also be produced by *S. aureus* to evade host immune responses. Together, these bacterial components act to ensure the survival of the bacteria within the host.

The fourth step to occur in bacterial pathogenesis is tissue destruction. This also involves a large number of bacterial virulence factors, as well as the host immune response. In gram positive bacteria, exotoxins are key virulence factors. Exotoxins are toxic bacterial proteins which are usually secreted into the medium by growing bacteria (Brock and Madigan, 1991). *Staphylococcus aureus* strains can produce many different exotoxins. Dermonecrotic toxin, enterotoxin and toxic shock syndrome toxin (TSST-1) are just three toxins which *S. aureus* isolates can produce to aid in the development of disease symptoms. Hydrolytic enzymes such as hyaluronidase and proteases are also produced. They can degrade extracellular matrix components and thus disrupt host tissue structure. Hydrolytic enzymes are also beneficial to the bacteria because they provide

carbon sources and energy to the bacteria by breaking host polymers into usable low-molecular-weight sugars and amino acids (Salyers and Whitt, 1995). *Staphylococcus aureus* can also use enzymes such as DNAses to reduce the viscosity of debris from dead host cells, thus allowing spread of the bacteria.

Bacterial cell walls are often associated with host tissue damage. Peptidoglycan is the main structural polymer in the staphylococcal cell wall (Brock and Madigan, 1991). Peptidoglycan is endotoxin-like in that it is pyrogenic, it activates the host complement cascade and it generates chemotactic factors which can result in the aggregation and lysis of blood components (Salyers and Whitt, 1997).

It is evident that *Staphylococcus aureus* has a diverse arsenal of components and products that contribute to the pathogenesis of infection. Every strain of *S. aureus* may not possess all of these virulence factors, but it should be noted that when these components and products are present or being expressed, they may have overlapping roles and can act either in concert or alone (Lowy, 1998).

1.1.2. Diseases

Staphylococcus aureus is the causative agent of many diseases. The majority of *S. aureus* infections are acute and referred to as pyogenic or pus-eliciting, but the organism does cause other types of infections. Some of the common ailments caused by *S. aureus* are: pyodermas, scalded skin syndrome, nephritis, food poisoning, endocarditis, pneumonia, arthritis, post-surgical wound infections and bacteremia (Projan and Novick, 1997; Brock and Madigan, 1991). Because of the large number of virulence factors *S.*

aureus can possess and express, it is possible for all of these conditions to occur. Although *S. aureus* is a pathogen, it is not always harmful and can be part of the normal flora of humans. Up to thirty percent of the human population has *S. aureus* on their skin with carriage being particularly common in healthcare workers (Siu, 1994). These people are classified as symptomless carriers and appear to be healthy individuals, but are a reservoir of the bacteria allowing for spread to others and possible harmful infection of themselves at a later time.

1.1.2.1. Glomerulonephritis

Glomerulonephritis has often been reported to occur after staphylococcal bacteremia and is often associated with endocarditis (reviewed in Yoh et al., 1997). Staphylococcal infection may also play a role in some forms of primary glomerulonephritis (Sato et al., 1979; Spector et al., 1980). Glomerulonephritis findings can include glomerular lesions associated with visceral abscesses caused by the staphylococcal infection and immune complex deposits within the kidney (Spector et al., 1980). Staphylococcus enterotoxins are known to act as superantigens and have been reported to be involved in the pathogenesis of glomerulonephritis following staphylococcal infection (Koyama et al., 1995). This results in the over activation of host T-cells and the excessive production of cytokines which can lead to a variety of symptoms including shock (Salyers and Whitt, 1995).

1.1.2.2. Bacterial Arthritis

Infectious arthritis, septic arthritis and suppurative arthritis are all comparable terms that imply an infectious pathogen, such as bacteria, and is responsible for an inflammatory reaction in the joint (Gentry, 1997). The development of bacterial arthritis is a potentially life-threatening condition that requires prompt medical attention. Bacteria may spread to the blood and individuals can become septicemic. *Staphylococcus aureus* is the most common pathogen causing non-gonococcal bacterial arthritis and accounts for some 80% of cases (Mikhail and Alarcon, 1993). The synovium of joints is a highly vascular connective tissue without a basement membrane and, thus is susceptible to hematogenous seeding of bacteria (Gentry, 1997). Bacteria can also be introduced into the joint through surgery or a penetrating wound. Once the bacteria are present in the joint, host inflammatory mediators are released in large amounts which leads to synovitis, with the potential for subsequent cartilage and subchondral bone destruction (Bremell et al., 1991). A number of *S. aureus* virulence factors have been investigated to determine their role in bacterial arthritis (Gemmell et al., 1997; Abdelnour et al., 1993; Cunningham et al., 1996). Factors such as α- and β- haemolysins, protein A, and coagulase are thought to be important in the pathogenicity of *S. aureus* in bacterial arthritis (Gemmell et al., 1997; Nilsson et al., 1999). The outcome of bacterial arthritis depends on multiple associated factors, with prognosis poor in certain groups (Goldenberg and Reed, 1985; Bremell et al., 1990). Risk factors include impaired host defense mechanisms resulting from the use of immunosuppressive drugs, chronic illnesses such as diabetes, as well as previous joint damage (Bremell et al., 1990; Goldenberg and Reed, 1985). Other

destructive joint diseases, such as rheumatoid arthritis, are related to an increased incidence of bacterial arthritis (Abdelnour et al., 1994; Cunningham et al., 1996). In general, if early diagnosis is made and treatment is initiated immediately, less sequelae will result.

1.1.2.3. Endocarditis

Endocarditis is defined as the inflammation of one or more heart valves and can be caused by a number a different pathogens. As with any condition involving the heart, endocarditis can be fatal because destruction of the valves and surrounding heart tissue can lead to heart failure (Salyers and Whitt, 1994). The prevalence of *S. aureus* endocarditis has increased and now accounts for 25 to 35 percent of cases in some locales (Sanabria et al., 1990; Sandre and Shafran, 1996). *Staphylococcus aureus* is an ideal endocarditis pathogen as the organism possesses a number of virulence factors which enables it to establish the disease (Ing et al., 1997). This disease occurs frequently in intravenous drug users, elderly patients, hospitalized patients and patients with prosthetic valves. Initially, endocarditis may present with fever and malaise therefore making diagnosis difficult and often missed (Marantz et al., 1987). Depending on underlying complications, endocarditis can be treated or be fatal as mentioned earlier. *Staphylococcus aureus* is also one of the most common pathogens in nosocomial and prosthetic-valve endocarditis (Lowy, 1998). Prosthetic-valve endocarditis is often fulminant and is characterized by the formation of myocardial abscesses and the

development of valvular insufficiency, often requiring surgery and replacement of the colonized valve.

1.1.3. Antibiotic Resistance

With time, and the increasing use of antibiotics, bacteria tend to adapt to their environment in order to survive (Jacoby et al., 1991). These steps in evolution tend to make the bacteria even more of a health risk than they already are and extremely difficult to control. Increasing bacterial resistance to many antibiotics that once readily cured bacterial diseases is cause for increasing concern. With each passing decade, bacteria that defy not only one, but multiple antibiotics have become increasingly common (Levy, 1998).

1.1.3.1. Methicillin-resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus developed resistance to penicillin via a plasmid and an extracellular enzyme called penicillinase. Penicillinase, or also referred to as β-lactamase, hydrolyzes the β-lactam ring of the penicillin thereby making it inactive (Labischinski, 1992). Methicillin, a semisynthetic derivative of penicillin not susceptible to penicillinase, was then developed to treat *S. aureus* infections and subsequently, methicillin-resistant *Staphylococcus aureus* (MRSA) have appeared. First identified in 1961, MRSA, which is also referred to as multiple resistant *S. aureus*, is emerging as a problem in countries all around the world (Akram and Glatt, 1998; Voss et al., 1994; Dunford, 1997). Initially, MRSA was mainly a problem among adults, but now is highly prevalent in pediatric populations (Boxerbaum et al., 1988). Colonization and/or

infection with MRSA tends to occur in seriously ill patients who have compromised defense mechanisms or require intensive care, broad spectrum or high-dose antibiotic therapy and frequent or prolonged hospital exposure (Boxerbaum et al., 1988). In some hospitals, more than sixty percent of *S. aureus* strains are resistant to methicillin and many of these are cleared only by glycopeptide antibiotics, like vancomycin (Levy, 1998). This development is not only a threat to lives but is also a huge financial cost on society.

1.1.3.2. Emergence of MRSA

Staphylococci become resistant to methicillin through the acquisition of a chromosomal gene (*mecA*) which encodes an alternative target that is not inactivated by β-lactams (Jacoby and Archer, 1991). The *mecA* gene encodes a penicillin-binding protein (PBP), PBP2a, that has a low affinity for β-lactams and that can substitute for the function of other β-lactam-susceptible PBPs in the bacterial cell wall (Archer and Niemeyer, 1994). The exact origin of the *mecA* gene is unclear, but there have been two main speculations. The first speculation involves horizontal transfer and a multiclonal theory where PBP2a is adapted from other bacteria or even genera (Labischinski, 1992; Archer and Niemeyer, 1994; Archer et al., 1994). In comparing PBP of different species, PBP2a of *S. aureus* resembles PBP5 and PBP3 of *Enterococcus hiriae* and PBP2 and PBP3 of *Escherichia coli* (Piras et al., 1993). The 2 kb *mecA* gene and flanking DNA are unique to MRSA with no allelic equivalent found in methicillin-susceptible *S. aureus* (MSSA), a finding which also suggests horizontal transfer as the route of gene acquisition (Beck et al., 1986). The second theory is one that follows vertical transfer and

subsequent evolution. Some MRSA appear to be descendants of a single clone and through time have formed a single, related lineage of MRSA (Musser and Kapur, 1992). Although, the first theory of horizontal transfer seems to have a stronger case, neither theory has been fully proven or refuted.

1.1.3.3. Future Concerns - Emerging Resistance

Since the emergence of MRSA, the glycopeptide vancomycin has been the only uniformly effective treatment for staphylococcal infections. As of 1996, four clinical cases of *S. aureus* with reduced susceptibility to vancomycin were identified worldwide (Smith et al., 1999). Emergence of forms lacking sensitivity to vancomycin, called glycopeptide-intermediate *Staphylococcus aureus* (GISA), signifies that variants untreatable by every known antibiotic may be evolving.

1.1.3.4. Current Treatments

Treatment of *S. aureus* infections should be based on the antimicrobial susceptibility profile of the organism. Penicillin remains the drug of choice if the isolate is sensitive to it, but 70 to 80 percent of *S. aureus* isolates are resistant to penicillin (Atkinson and Lorian, 1984). A semisynthetic penicillin such as methicillin is indicated for β-lactamase producing strains. Vancomycin is the drug of choice for MRSA isolates. The glycopeptide-intermediate strains reported to date have been sensitive to chloramphenicol, gentamicin, rifampin, trimethoprim-sulfamethoxazole, and tetracycline (Hiramatsu et al., 1997; Tenover et al, 1998). Antibiotic treatment in parallel with

surgical debridement may be necessary to treat cases involving GISA strains (Fowler et al., 1998; Ingerman and Santoro, 1989). Prolonged exposure of a patient to one specific antibiotic should be avoided to inhibit the development of antibiotic resistance, a sequelae which further complicates treatment options. Antimicrobial combinations have been used in some cases to increase bactericidal activity and to prevent the emergence of resistance (Chambers, 1997).

1.2. PLASMINOGEN ACTIVATORS

Plasminogen activators have an ubiquitous distribution in the body, usually in low concentrations. The inactive enzyme, plasminogen, is converted to an active proteinase, plasmin, by the action of plasminogen activators such as urokinase (UK). The end-product of this reaction, plasmin, is a trypsin-like endopeptidase responsible for the degradation of fibrin (Christman et al., 1977). Plasmin substrates include casein, cell membrane proteins, immunoglobulins, complement components and factors from the clotting cascade. With the introduction of plasmin, the complement system can become activated and an accumulation of polymorphonuclear cells (PMNs) occurs through the generation of C3a. Mammalian plasminogen activators can be divided into two main groups, urokinase plasminogen activator (u-PA), which is the most abundant, and tissue plasminogen activator (t-PA) (Hart and Rehemtulla, 1988). Urokinase plasminogen activators are the primary plasminogen activator in connective tissues, including skin, and is expressed by inflammatory cells such as macrophages and PMNs, whereas t-PA is the primary fibrinolytic enzyme of the vasculature (Hart, 1992; Hart and Woods, 1994). Both

activators activity are tightly regulated by cytokines, growth factors and by specific inhibitors (Zavizion et al., 1997).

1.2.1. Urokinase Plasminogen Activator

Urokinase was first described by Sahli in 1885 and subsequently has been purified from human urine, allowing for further investigations of the protein. It is synthesized as a single chain protein which must be proteolytically cleaved to yield an active enzyme. Active enzyme is comprised of two subunits, one with catalytic activity and another non-catalytic, and can be found in two forms, one of high molecular weight (54 700 kDa) and a lower molecular weight form (31 500 kDa) (Christman et al., 1977). The kidneys have a high concentration of UK because of the rich blood supply and a complicated network of tubules to keep functional (Wagner et al., 1996). Urokinase is actively synthesized by renal tubule cells. Another organ which UK is of importance is in the lung. Here, UK assists in the maintenance of well aerated alveoli and prevention of fibrin deposition within the lung (Hart, 1992).

1.2.2. Bacterial Plasminogen Activator-like Proteins

Many clinical strains of *S. aureus* produce a bacterial PA-like protein staphylokinase, and acquire host plasminogen which can then be activated by u-PA or t-PA (Christner and Boyle, 1996). Staphylokinase is not an enzyme, but a protein which forms a complex with plasminogen leading to direct activation of the enzyme. The organism then uses the activated complex for growth and invasion (Zavizion et al., 1997; Boyle and Lottenberg, 1997). Hart and Woods (1994) demonstrated that specific host

proteinases such as UK can stimulate the *in vitro* growth of blood-derived microorganisms especially when the initial inoculum is low. Urokinase enhanced the growth of some gram-negative bacteria, gram-positive bacteria and stimulated the growth of yeast (Hart and Woods, 1994). Examination of a larger panel of clinical isolates of *S. aureus* revealed that some isolates were responsive to UK enhancement of growth and others were not (Hart et al., 1996). Only 1/26 Canadian MRSA isolates (isolate 456) were UK responsive, whereas over fifty percent of MSSA were UK responsive. MRSA 456 (isolated from a rectal swab) was one of two isolates harvested from a patient and responded to as few as 50 U UK/ml within 9 hours of incubation. The other isolate, MRSA 457, removed from a hip abscess, was consistently nonresponsive to UK even though it was also a MRSA. Urokinase responsiveness *in vitro* thus defines a subset of both MRSA and MSSA isolates.

1.3. ANIMAL MODELS

Animal models provide an *in vivo* means of testing and incorporating those variables not present *in vitro*. Models are developed to mimic specific diseases or specific disease situations. With the use of animals, studies on the mechanisms by which microorganisms infect and cause disease have been greatly facilitated.

1.3.1. *Staphylococcus aureus* Mouse Models

Many animal models have been developed to study different *S. aureus* infections. The problem of obtaining meaningful infection models have been

compounded by the resistance of test animals to challenge with these organisms (Adlam, et al., 1983). In most circumstances, very large numbers of bacteria are required to establish infections. The route of inoculation has an impact on the outcome of the disease. When comparing routes of inoculation using the same strain, it has been reported that infection by the intracardiac, intraperitoneal, intracranial, intrahepatic and intrasplenic routes resulted in the most deaths, with deaths occurring more rapidly than other routes (Smith et al., 1960). Interestingly, the events preceding death were similar regardless of route of challenge.

1.3.1.1. Kidney Abscess Animal Model

S. aureus injected intravenously appeared in the urine of test animals, not from secretion or excretion by the kidney, but from the formation of lesions in the kidney (Dyke, 1923). It has been suggested that the production of coagulase by *S. aureus* might allow clumping and coagulative necrosis to occur in the kidney which would prevent passage through into the urine (De Navasquez, 1950; Smith and Dubos, 1956). However, some coagulase-negative mutants behave in a similar manner to coagulase producing strains, thus coagulase production alone could not be responsible for kidney abscess formation (Li and Kapral, 1962). *Staphylococcus aureus* α-toxin is also thought to contribute to colonization of the kidney, as α-toxin negative mutants failed to multiply in the kidney although initial lodgment numbers were comparable to α-toxin positive strains (Kapral, 1974). Gorrill found the ID_{50} by the intravenous route to be approximately 10^6 and the number of organisms required to infect the mouse kidney was not reduced by

animal passage (Gorrill, 1958). Intravenous challenge with more than one *S. aureus* strain demonstrated that individual kidney abscess foci were produced by individual strains (Gorrill, 1958).

Lee and colleagues used a mouse abscess model to study the role of the mucoid phenotype on the pathogenecity of *S. aureus* strains (Lee et al., 1985). Following inoculation of C57Bl mice with mucoid and non-mucoid strains, all organs were cleared of the bacteria except the kidneys. The mucoid strain induced evident renal abscesses, whereas the non-mucoid strain induced minimal abscess formation (Lee et al., 1985). Interestingly, further studies with just the non-mucoid strain showed renal lesions began to resolve after one week post-inoculation and by day 24 approximately eighty percent of mice had sterile kidneys (Lee et al., 1989).

1.3.1.2. Bacterial Arthritis Animal Model

Controlled studies of bacterial arthritis in humans are obviously difficult to undertake so animal models have also been developed to perform research in this area. There are two different methods of bacterial inoculation that have been employed to induce bacterial arthritis in animal models. Bremell and colleagues developed an arthritic mouse model using an intravenous administration of 10^7 cells of *S. aureus* into the mouse tail (Bremell et al., 1991). This protocol resulted in no detectable septicemia, indicating that the bacteria remain in the blood for a short time. The bacteria were also shown to colonize the spleen and kidney (Bremell et al., 1991). Other attempted routes (ie:

intraperitoneal and subcutaneous) of inoculation did not lead to arthritis and the disease process required the injection of live microorganisms.

The second animal model of bacterial arthritis involves injection of the knee joint with the antigenic components of bacteria or whole bacteria (Keystone et al., 1977; Bremell et al., 1991). Within 48 hours after inoculation, the injected joints were swollen and arthritis was evident. This model may resemble infections obtained from joint penetration, but the hematogenous route of infection is the most common cause of bacterial arthritis, and hence direct injection of the joint is the least popular of the two models presented.

1.3.2. Urokinase Plasminogen Activator Deficient Mouse

When developing an animal model for research, genetic manipulation can often be useful. Animals with a loss of a gene, or knockouts, first must be examined for any side effects of the genetic disruption before further experiments can be confidently performed with them as the animal model. Urokinase plasminogen activator deficient mice (uPA-/-) lack uPA at the genomic, mRNA, protein and enzyme activity levels (Carmeliet et al., 1994). This allows the role of uPA to be evaluated without interfering with any other normal activities.

Urokinase deficient mice have a normal phenotype under non-stressful conditions (Carmeliet et al., 1994). They appear to live a normal mouse lifespan and reproduce as per usual. However, some infections such as *Cryptococcus neoformans* and botryomycosis have been shown to be more detrimental to uPA-/- mice than to uPA+/+

controls (Gyetko et al., 1996; Shapiro et al., 1997). The protozoan pathogen *Pneumocystis carinii* also requires uPA for clearance. Following inoculation with *P. carinii*, mice deficient of UK developed heavy pneumonia and exhibited decreased accumulation of inflammatory cells when compared to positive control mice (Beck et al., 1999). Wound healing is also impaired in urokinase deficient mice. These findings imply that although the uPA-/- mice appear "normal" they may be more susceptible to certain infectious conditions.

1.3.3. Gender Differences

Sex hormones have been known to influence infections, sometimes making one gender more susceptible to a pathogen than the other gender. Androgens and estrogens can act in either a positive or negative manner in the development of a disease. Testosterone has been shown to act synergistically with antibiotics to prevent or clear infections and has been associated with the prevention of paralysis in rats inoculated with herpes simplex virus (Atef and Sokkar, 1980; Yirrell et al., 1987). Some early reports have indicated that sex hormones may influence bacterial growth and survival directly (Warren et al., 1970), but this remains to be clarified with physiological levels of hormones. Estrogens have a tendency to be more detrimental to the host in the development of infections (Kinsman and Collard, 1986; Harle et al., 1975), but studies have also shown the contrary (Best et al., 1986). Gender is known to influence inflammatory and immune responses (reviewed in Yung, 1999) and gender differences in

the regulation of fibrinolysis have also been reported (reviewed in Hart, 1992) so gender considerations may be operative at multiple levels.

1.4. RATIONALE FOR STUDY

To conclude, *Staphylococcus aureus* remains a prominent microorganism causing disease. With a wide range of virulence factors and the development of antibiotic resistance, treatment of *S. aureus* infections is becoming more challenging. MRSA isolates are becoming more prevalent and strains of GISA have now surfaced. It is thought that the trend of antibiotic resistance will continue, possibly taking us back to a situation like the pre-antibiotic era.

Plasminogen activators such as UK have been shown *in vitro* to enhance the growth of some strains of bacteria including MRSA and MSSA isolates. Plasminogen activator-like proteins, such as staphylokinase, can be utilized for growth and invasion. When formed, plasmin can degrade tissue matrix therefore allowing the bacteria to cross barriers which are otherwise impermeable. The question then arises if the bacteria, specifically *S. aureus*, can use host UK *in vivo* in the pathogenesis and development of disease?

Previous animal models involving *S. aureus* have reported the development of a range of diseases. Animal models have been used to study endocarditis, bacteremia, joint inflammation, kidney disease and a range of other ailments. The gender of animals can influence the onset of disease depending on the causative organism and the animal model utilized. In some situations females may be more susceptible to infection and in

others the male species is more prone to infection. Therefore some of these variables will be investigated in the animal model to be used in this study.

1.5. HYPOTHESIS AND OBJECTIVES

The principal hypotheses are: 1) *in vitro* UK responsiveness of specific isolates will influence *S. aureus* pathogenesis in mouse models and correlate with *in vitro* findings, and 2) there will be a gender difference in the susceptibility to infection with *S. aureus*.

The objectives of this thesis are to develop an animal model involving *S. aureus* infection and use this animal model to: 1) determine if *in vitro* bacterial UK responsiveness has an overt effect on *in vivo* pathogenesis; 2) compare MRSA isolates with MSSA isolates with regard to pathogenesis and; 3) determine if there is a gender difference in susceptibility to *S. aureus*. The primary aims of the investigations presented are:

1. To develop an animal model to study *S. aureus* infection and characterize the natural history of the infection.
2. To assess the survival rate and bacterial colonization of mice inoculated with *in vitro* UK responsive and UK non-responsive isolates of MRSA and MSSA.
3. To determine the influence of gender on the development of disease by *S. aureus* through mortality and colonization studies.
4. To analyze histological changes of affected organs in the different treatment groups.

CHAPTER 2

MATERIALS AND METHODS

2.1. BACTERIAL ISOLATES

Table 1 lists the clinical isolates of *Staphylococcus aureus* used in the current studies. Methicillin-resistant *Staphylococcus aureus* 456 was isolated from a rectal swab of a patient, while MRSA 457 was isolated from a hip abscess of the same patient. For MSSA isolates, HR78 was isolated from sputum and CSA-1 was isolated from a hand abscess of two different patients. Both MRSA 456 and MSSA HR78 show enhanced bacterial growth when urokinase is added to the in vitro growth medium (Hart et al., 1996). Methicillin-resistant *Staphylococcus aureus* 457 and MSSA CSA-1 show no enhanced growth response to urokinase in vitro. All of the isolates were obtained through the Office of Infection Control at the Calgary General Hospital - Bow Valley Centre (Calgary, Alberta) and have been used in previous studies (Hart et al., 1996).

Table 1. Features of MSSA and MRSA clinical isolates

Staphylococcus aureus isolate	Isolate source	In vitro urokinase responsive
MRSA 456	rectal swab	yes
MRSA 457	hip abscess	no
MSSA HR78	sputum	yes
MSSA CSA-1	hand abscess	no

2.1.1. Culture Conditions and Inoculum Preparation

Bacterial cultures were stored at -70°C using clearly labeled Microbank™ vials (Pro-Lab Diagnostics, Richmond Hill, Ontario). Microbank™ is a sterile vial containing porous beads which serve as carriers to support microorganisms. When a fresh culture was required, a single bead was aseptically removed from the appropriate vial and used directly to streak trypticase soy agar (DIFCO Laboratories, Detroit, MI). The plate was then incubated at 37°C overnight and the culture was checked for purity. A few single colonies of bacteria were then inoculated into 1/10 tryptic soy broth (DIFCO Laboratories, Detroit, MI) in minimal salts medium (M9) with glucose and grown overnight in a shaking water bath (50 oscillations/minute) at 37°C. Samples were then alternately centrifuged and washed with cold sterile PBS (pH 7.2) for a total of three cycles. Centrifugation was for 10 minutes at 4°C and 7000 x *g* in a Sorvall Superspeed centrifuge. The final bacterial concentrates were resuspended in 5ml of sterile PBS, and aliquoted to be stored at -70°C. The number of organisms in the samples was determined by standard dilution technique and plate counts.

2.2. ANIMAL MODEL

Two strains of mice were used as animal models in these studies. The mice utilized were, male and female CD1 mice, obtained from Life & Environmental Sciences Animal Resource Centre, University of Calgary, Calgary and male and female uPA-/- mice originally obtained from Dr. Peter Carmeliet at The University of Leuven, Belgium. Prior to inoculation, bacterial harvests were thawed, serially diluted and plated out on

mannitol salt agar plates (DIFCO Laboratories, Detroit, MI) to quantify viable organisms. The bacterial concentrates were then diluted with sterile PBS (pH 7.2) to the appropriate concentration (10^8 CFU/ml) to give an inoculum of approximately 10^7 CFU per mouse in a volume of 100µl. Most of the experiments performed used an inoculation dose of 10^7 CFU per mouse. Because an exact CFU count is difficult to obtain, the inoculation dose ranged from approximately $9x10^6$ CFU/mouse to $2x10^7$ CFU/mouse. Inoculum dosage for each experiment was recorded. This dilution was found to be optimal in preliminary experiments. Depending on the mouse strain, doses of organisms less than 10^7 CFU/mouse induced inconsistent or no chronic infection, whereas a dose of 10^8 CFU/mouse resulted in rapid illness and mortality in a high percentage of the mice. In the dose response experiments in uPA-/- mice, the appropriate dilutions were made to achieve the desired inoculation dosage in a volume of 100µl. Injections were administered intravenously via the tail using a sterile 30 gauge needle. Upon the completion of inoculations, the bacteria were again plated to verify the concentration of the inoculum.

Mice were housed in groups determined by infectious agent, in enclosed shelved cabinets with filtered air flow. They were given water *ad libitum* and fed a pelleted commercial rodent diet. Animals were maintained in accordance with the *Guide to the Care and Use of Experimental Animals* approved by the Canadian Council for Animal Care and the use of mice was reviewed and approved by the University of Calgary animal care committee.

2.2.1. Castration of CD1 mice

Three week old male CD1 mice were anesthetized and surgical castration performed using an abdominal approach (Olson and Bruce, 1986). Incisions were sutured and tissue glue applied to close the skin. All of the mice were closely monitored until complete recovery (>95% survival) and maintained until 8-10 weeks of age. Animals were also assessed at the time of sacrifice to confirm completeness of the operation.

2.3. ASSESSMENT OF INFECTION

Mice were monitored on a daily basis for signs of illness and sacrificed immediately if they appeared to be under extreme stress (mice sacrificed at grade 3, assessed with a pre-set scoring system, Table 2.). At the time of sacrifice, mice were anesthetized and bled by cardiac puncture to collect serum. Serum was clearly labeled and tested for Murine Hepatitis Virus (MHV), as presence of MHV may complicate the experimental results. The serum was then frozen at -70°C. Whole organs such as the liver, lung, spleen, heart and kidney were routinely aseptically harvested, placed in sterile PBS and weighed. Joints such as the knee and elbow were also removed in certain experiments and treated in the same manner. Organs were removed in a manner to reduce contamination by any other tissues such as adipose. The harvested knee consisted of a portion from the mid-femur to mid-tibia/fibula and the elbow sample was from the mid-humerus to mid-radius/ulna. In addition to visual observation of the tissues, quantitative bacteriology was performed on each tissue and/or joint sample. Whole samples were homogenized under sterile conditions, using a Polytron PT 2100

homogenizer (Kinematica AG Dispersing and Mixing Technology, Switzerland) at 24 000 rev/min for approximately 30 seconds. On occasion, joint samples required longer durations of homogenizing. During this time, all samples were kept on ice and the homogenizer was washed thoroughly and sterilized after every sample. Individual organ or joint weight was determined and homogenates serially diluted in sterile PBS and plated out on mannitol salt agar to determine bacterial counts per gram of corresponding sample. Routinely, four serial dilutions per sample were spot plated and average CFU counts calculated with the growth from as many dilutions as possible. Dilutions with extreme bacterial growth were considered to have too many bacterial colonies to count (TMTC). Only those dilution spots with distinct single colonies were used for calculations. Experimental groups started the procedures with a 'n' value that would allow for statistical analysis. The 'n' of an experimental group for most experiments was $n=10\pm2$. In repeat experiments with a higher chance of mortality, the number of animals/group was raised accordingly (n=15-25).

Bacterial counts were expressed as $\log_{10}$ transformed mean values and SD per gram of tissue. Statistical analysis of bacterial colonization differences between male and female mice, MRSA 456 and MRSA 457 colonization, and CSA-1 and HR78 colonization was determined by Student's *t* test. Fisher's test was used to compare the proportion of chronic colonization between the different genders. The significance level was established at $P<0.05$.

Table 2. Scoring system for experimental mice

Grade	Symptoms
Grade 0	-healthy animal
Grade 1	-floppy tail
Grade 2	-floppy tail
	-fur is not well maintained
Grade 3	-floppy tail
	-hyporesponsive when touched
	-incontinence
Grade 4	-moribund

2.4. HISTOLOGY

At different time points post-inoculation such as two weeks, four weeks, and six weeks, kidneys and knees were aseptically removed for histological processing and fixed in 10% neutral buffered formalin. Paraffin and methyl methylacrylate (MMA) embedding were performed and blocks sectioned using standard methodology. Paraffin sections were stained with hematoxylin and eosin (H&E) or Gram stain, and Lee's methylene blue-basic fuchsin was used on the MMA sections to highlight bacterial colonies (Bennett et al., 1976). Prepared slides were analyzed for the extent of inflammatory cell infiltration, bacterial colonization and tissue destruction. Samples from different time points, different mice strains and from mice inoculated with different bacterial isolates were compared.

CHAPTER 3

RESULTS

3.1. CD1 MOUSE MODEL

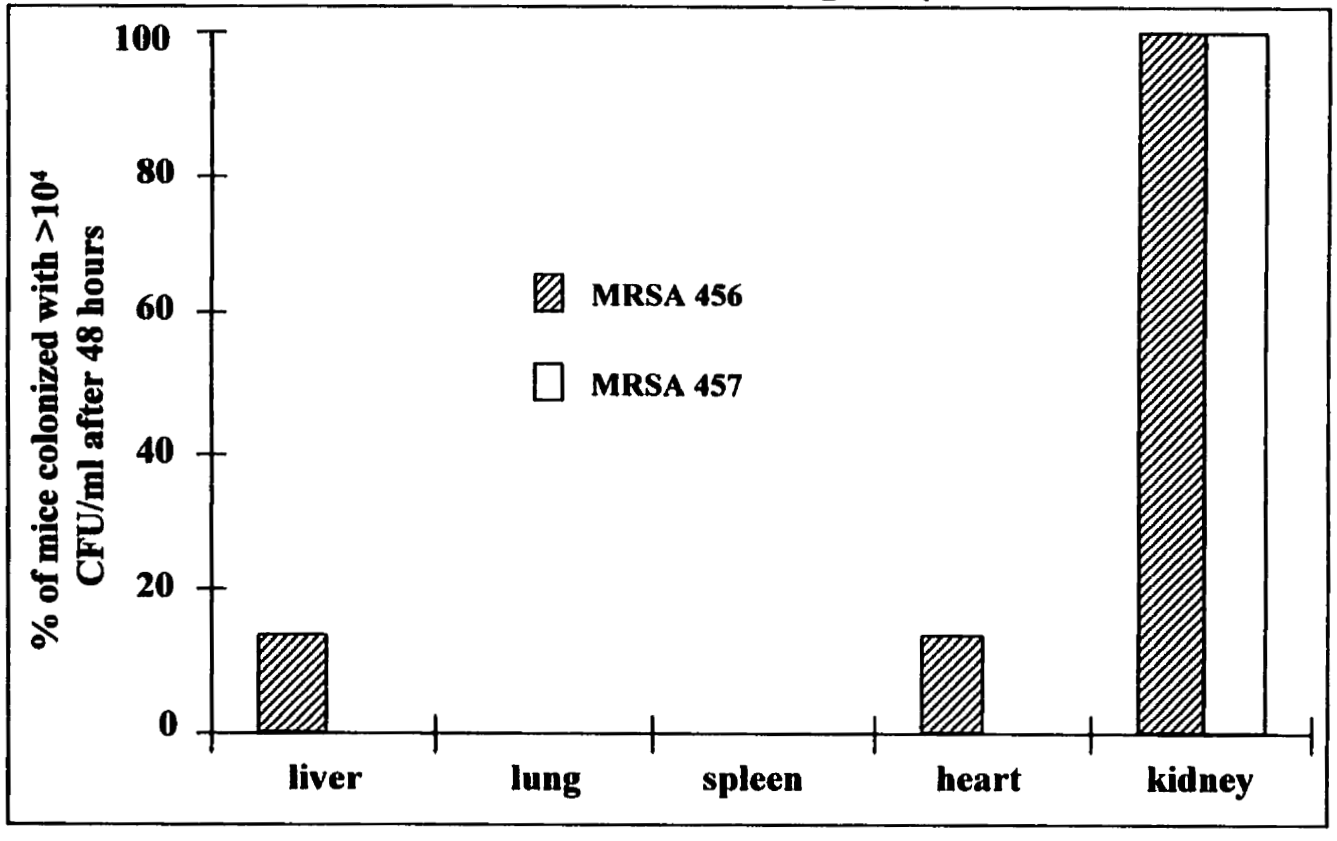
3.1.1. Organ Colonization

To assess the establishment of infection following intravenous *S. aureus* inoculation of CD1 mice, the colonization of different mouse organs was analyzed 48 hours post-inoculation. Organs with bacterial counts greater than 0.1% of the inoculum were considered as colonized. Inoculation of male and female CD1 mice with MRSA resulted in 100% colonization of the mouse kidneys (Fig. 1). Minimal colonization of the liver and heart were observed following MRSA 456 inoculation, but not with MRSA 457 inoculation, and no colonization of the lung and spleen were ever observed. Patterns of colonization for MSSA isolates were similar to those following MRSA inoculation, with the kidney again being the predominant organ colonized (data not shown). If a bacterial count lower than 10^4 (0.1%) defined colonization, the results would be similar. If one bacterial colony in the neat sample, deemed an organ colonized, the kidney would still be the predominant organ colonized, with heart and liver colonization increasing by a maximum of 20%. Mouse organs not colonized with □0.1% of the inoculum, generally showed no viable bacteria in the tissue. Bacteria that are passing through an organ following inoculation, eventually to be cleared, would not show a CFU count of 0.1% of the initial inoculation dose at 48 hours post-inoculation.

Figure 1. Pattern of colonization of CD1 mouse organs by MRSA

CD1 mice were inoculated with 10^7 CFU of either MRSA 456 or MRSA 457. After 48 hours, the mice were sacrificed and organs harvested and analyzed for *S. aureus*. Organs with bacterial counts greater than 0.1% of the inoculum were considered as colonized.

Figure 1. Pattern of colonization of CD1 mouse organs by MRSA



Visual observations of the kidneys inoculated with MRSA or MSSA, often showed the development of abscesses on the surface of the organ, with abscess prevalence increasing over time (Fig. 2).

3.1.2. Chronic MRSA Infection

The CD1 mouse model used in this study was shown to lead to chronic kidney infection, where inoculation of mice with either MRSA or MSSA failed to show clearance of the inoculated bacteria up to 42 days post-inoculation (Fig. 3). Along with kidney colonization, it was shown that the bacteria were being shed into the bladder, as the bladder of chronically infected mice contained *S. aureus* (data not shown). The finding of predominantly renal colonization is of interest since this organ is a site of urokinase synthesis (reviewed in Hart, 1992). Thus, the animal model developed in this study is similar to previously reported renal abscess models (Lee et al., 1985 and Adlam et al., 1983) where chronic colonization by both MRSA and MSSA occurred, with the kidney being the predominant organ colonized.

3.1.3. Kidney Colonization by MRSA

Kidney colonization was analyzed further to determine if MRSA 456 and MRSA 457 behaved differently *in vivo* with respect to colonization. Both MRSA isolates tested exhibited similar patterns of colonization in the female CD1 mice. With an initial inoculation dose of 10^7 CFU/mouse, over 28 days post-inoculation the CFU/g of kidney tissue increased approximately one log value for both MRSA 456 and MRSA 457 (Fig.

Figure 2. Development of abscesses in the kidneys of chronically infected mice

The kidney on the right is from a female CD1 mouse infected with 10^7 CFU of MRSA 457, 4 weeks post-inoculation. The kidney on the left is from an age-matched control mouse.

Figure 2. Development of abscesses in the kidneys of chronically infected mice

Figure 3. Chronic colonization of CD1 male mice with MRSA 456

Male CD1 mice were inoculated with 10^7CFU of MRSA 456. Kidneys were harvested and analyzed for bacterial colonization up to 42 days post-inoculation. Of the colonized mice, bacterial clearance from the kidney was not evident up to 42 days post-inoculation. Data are $\log_{10}$ transformed averages and standard deviation of the means.

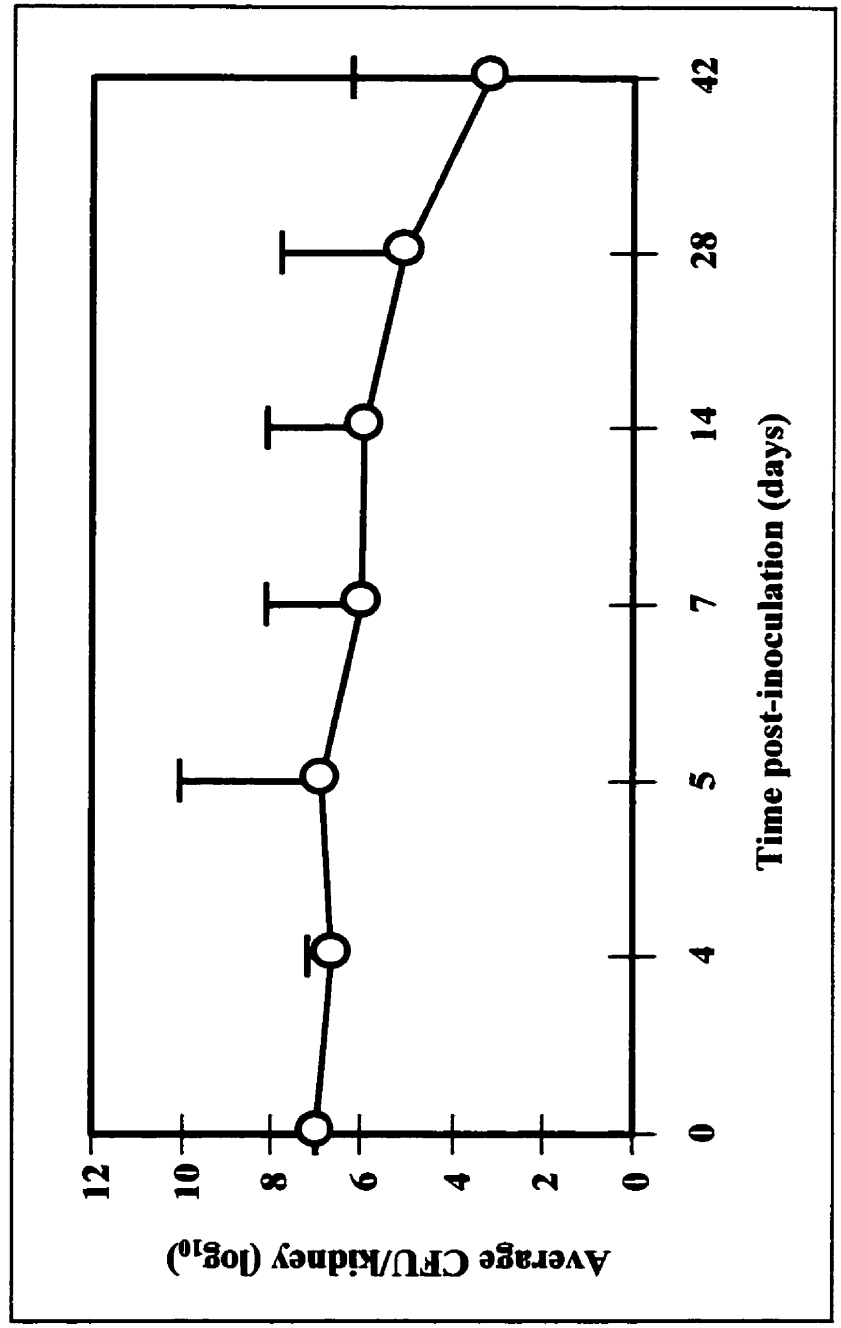


Figure 3. Chronic colonization of CD1 male mice with MRSA 456

4). The male CD1 mice showed similar results to those attained for the female mice (Fig. 5). MRSA 456 and MRSA 457 exhibited comparable colonization of the kidney at 14 days post-inoculation. Histological examination showed the presence of bacteria mainly in the tubules of the kidney along with infiltration of host inflammatory cells. There did not appear to be any obvious difference in histology between mice inoculated with MRSA 456 or MRSA 457.

3.1.4. Survival and Colonization of MRSA Inoculated Mice

Survival of MRSA inoculated mice was assessed to compare the *in vivo* behavior of MRSA 456 and MRSA 457 and to determine if a difference in susceptibility between male and female mice to MRSA exists. Male CD1 mice inoculated with either MRSA 456 or MRSA 457 had a 100% survival rate up to 28 days post-inoculation (data not shown). Female CD1 mice were more susceptible than male mice to MRSA lethality, following inoculation of either isolate of MRSA (Fig. 6). Inoculation of female mice with MRSA 456 resulted in a total of 8/28 deaths and inoculation with MRSA 457 resulted in a total of 7/27 deaths. Assessment of bacterial counts in the kidneys of surviving CD1 mice indicated that female mice displayed MRSA colonization levels 2 logs higher than the male mice colonized for the same period of time (Fig. 7). Female mice displayed significantly greater colonization than the male mice with MRSA 456 and MRSA 457 (P of <0.05). Following inoculation of MRSA isolates, there appeared to be a gender difference in CD1 mouse survival and colonization, but the two isolates of MRSA exhibited similar levels of colonization when the different isolates in the same gender of CD1 mice were compared (Fig. 7).

Figure 4. Colonization of female CD1 mice kidneys up to 28 days post-inoculation of MRSA

Female CD1 mice were inoculated with the indicated MRSA isolate and sacrificed at varying time points post-inoculation. The kidneys were harvested and processed for quantitative bacteriology. Data are presented as $\log_{10}$ transformed means +/- standard deviation of the means where n=10.

Figure 4. Colonization of female CD1 mice kidneys up to 28 days post-inoculation of MRSA

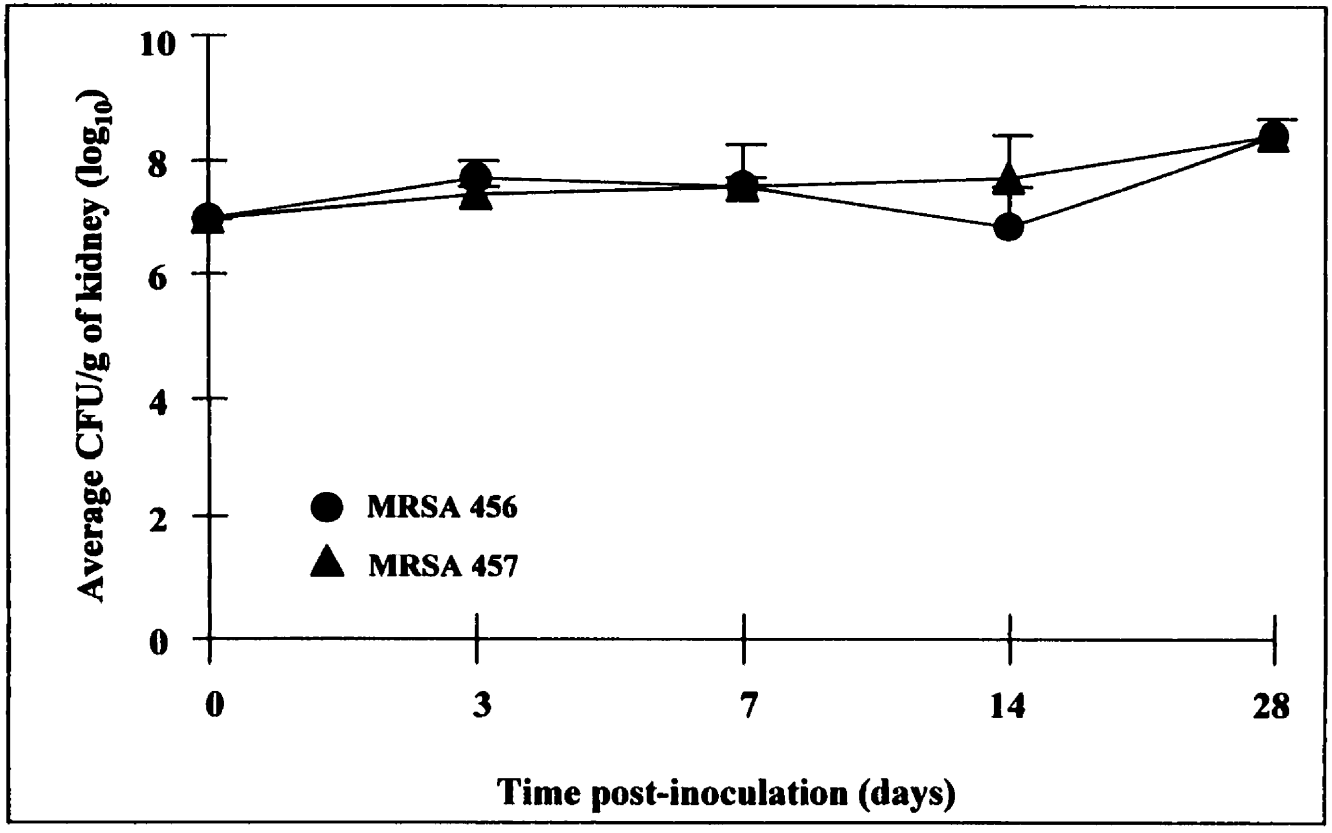


Figure 5. Colonization of male CD1 mice kidneys up to 14 days post-inoculation of MRSA

Groups of CD1 male mice were inoculated with either MRSA 456 or MRSA 457 and sacrificed at different time points post-inoculation. Kidneys were harvested and bacterial counts determined and expressed as CFU/g of kidney. Data are $\log_{10}$ transformed averages and standard deviation of the means where n=10.

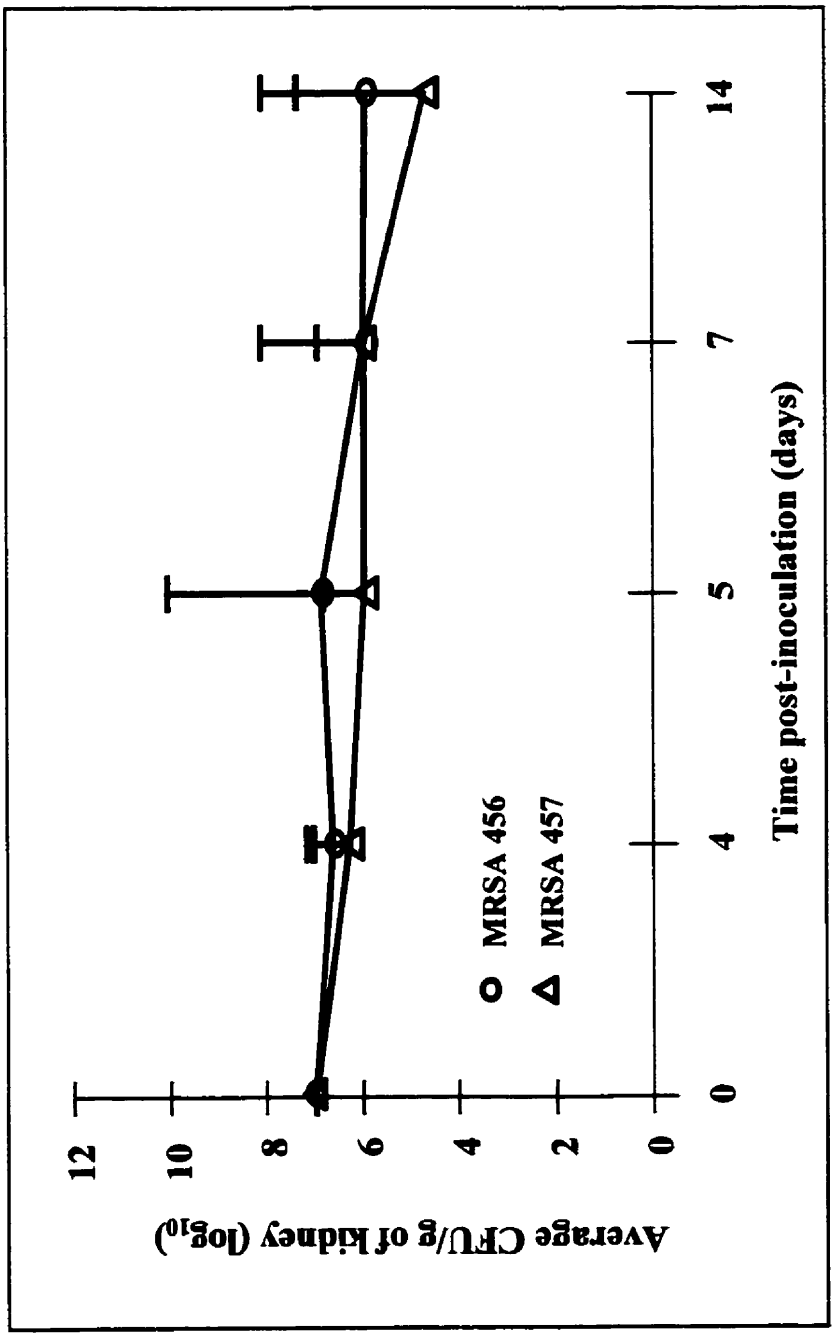


Figure 5. Colonization of male CD1 mice kidneys up to 14 days post-inoculation of MRSA

Figure 6. Mortality of CD1 female mice inoculated with MRSA 456 isolate or MRSA 457 isolate

Female CD1 mice were inoculated with 10^7 CFU and monitored up to 28 days post-inoculation. Each group began the experiment with 27-28 animals. At 3 days, 7days, 14 days and 28 days post-inoculation randomly selected female mice (4-7/ time point) were sacrificed and analyzed. Of the female survivors assessed at different time points, 100% showed kidney colonization.

Figure 6. Mortality of CD1 female mice inoculated with MRSA 456 or MRSA 457

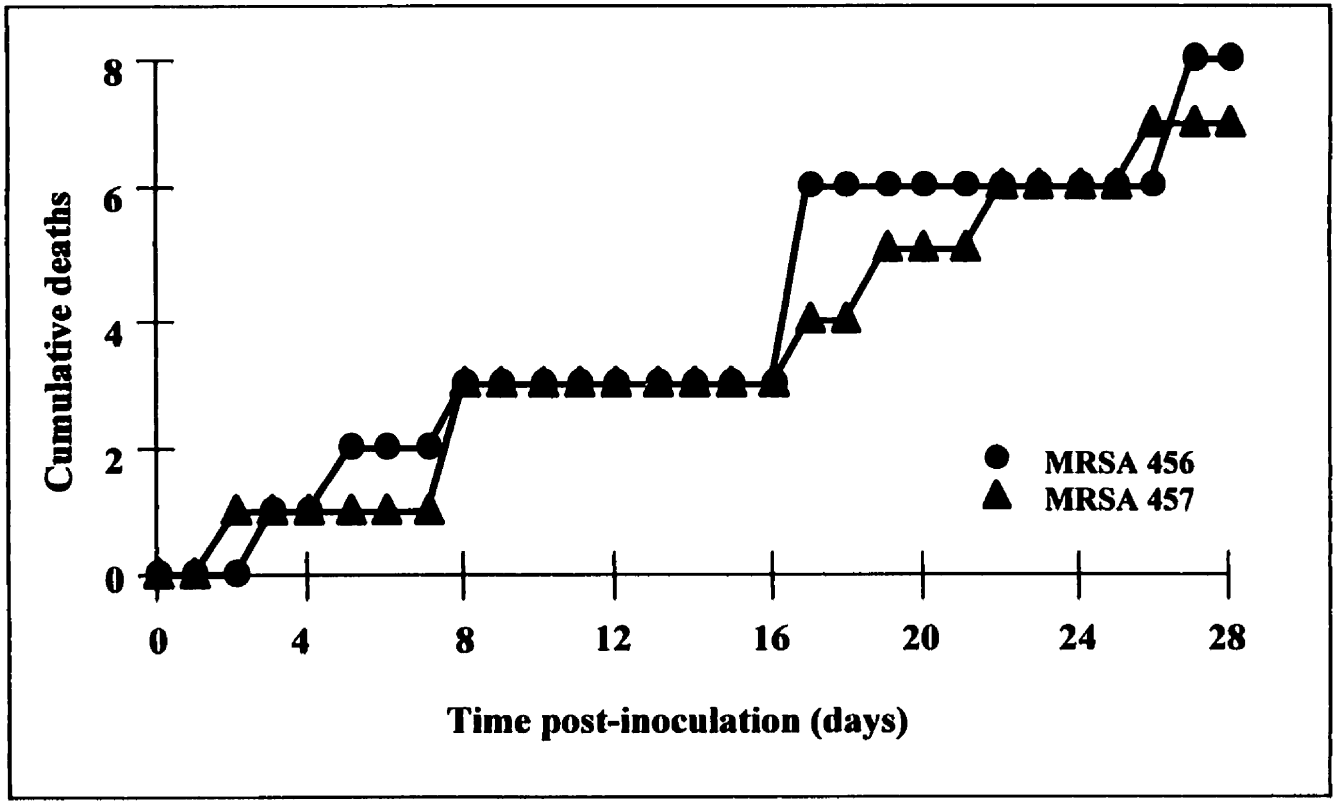
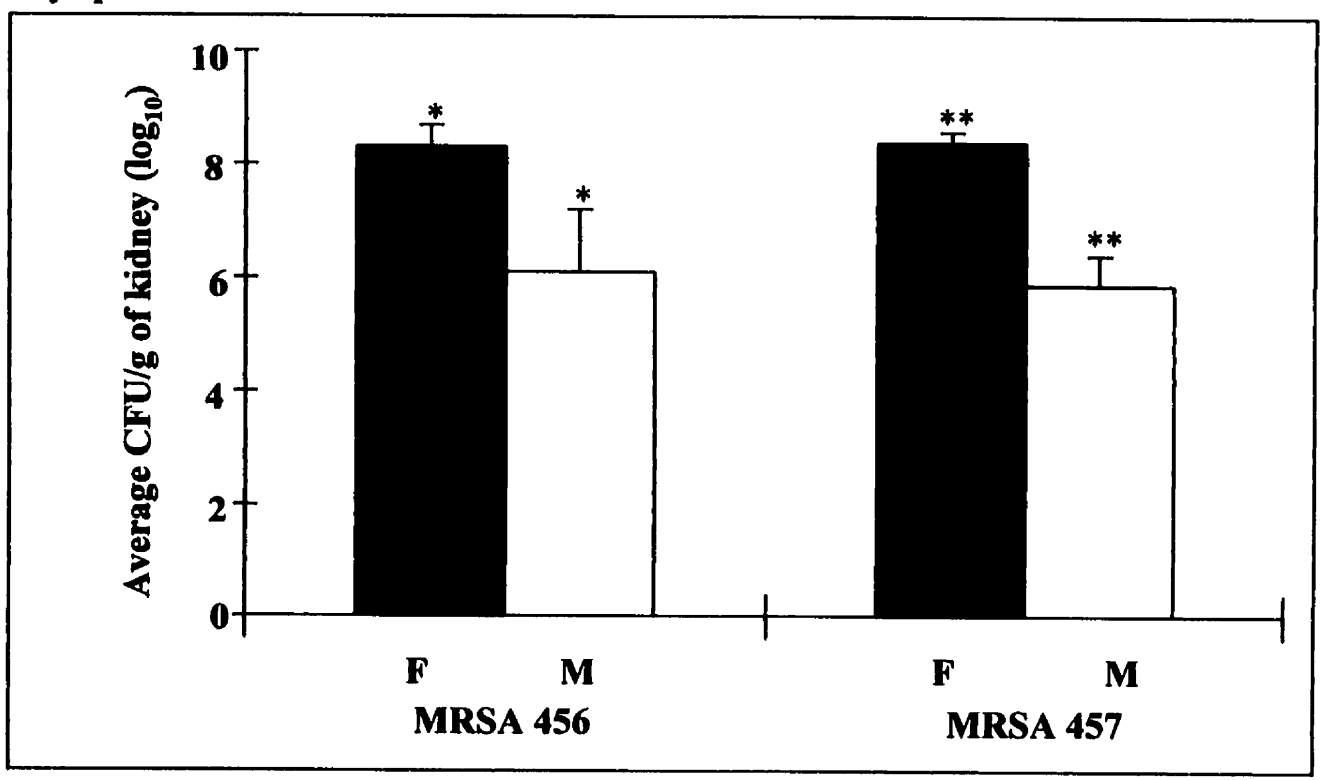


Figure 7. Colonization of the kidneys of male and female CD1 mice surviving to 28 days post-inoculation with MRSA

Colonization of the kidneys of male and female CD1 mice surviving inoculation with MRSA 456 or MRSA 457. Kidneys were harvested and analyzed for bacterial counts. Data presented are $\log_{10}$ transformed means +/- standard deviation of the mean. *, significant difference between male and female mice at P of <0.05. **, significant difference between male and female mice at P of <0.005.

Figure 7. Colonization of the kidneys of male and female CD1 mice surviving to 28 days post-inoculation with MRSA



3.1.5. Survival and Colonization of MSSA Inoculated Mice

Two MSSA isolates, HR78 and CSA-1, were used to compare the virulence of MRSA and MSSA isolates in the *in vivo* CD1 mouse model. The effect of gender on susceptibility to MSSA infection as well as the influence of *in vitro* UK responsiveness on *in vivo* growth were also evaluated. MSSA inoculation proved to be more lethal than MRSA to both male and female mice (Fig. 8 and Fig. 6). Female mice tended to die early in the experiment starting at day 2 post-inoculation, with mortality peaking around day 7. By 14 days post-inoculation, approximately 25% of the initial mice inoculated with either isolate of MSSA remained alive. Male mice inoculated with MSSA showed a more gradual trend of mortality, with a final survival outcome similar to the female mice. To examine the influence of MSSA isolates in the surviving mice, CFU/g of kidney tissue was calculated for these mice (Fig. 9). Female and male mice surviving to day 14 exhibited similar levels of kidney colonization and there was no evident difference between colonization by HR78 and CSA-1. Histological examination displayed no evident difference between mouse kidneys inoculated with MRSA 456 and MRSA 457 (data not shown).

3.1.6. Influence of Castration on Infection by MRSA

To further explore the influence of gender on MRSA colonization, CD1 male mice were castrated prior to sexual maturity and then inoculated with MRSA 456. Intact age-matched male and female mice were inoculated at the same time as the castrated

Figure 8. Mortality of CD1 mice inoculated with MSSA HR78 isolate or MSSA CSA-1 isolate

Groups of male and female CD1 mice (n=12) were inoculated with MSSA and observed for 14 days post-inoculation. Deaths were recorded daily. Fischer's test revealed no significant difference in mortality between groups inoculated with MSSA HR78 and those inoculated with MSSA CSA-1.

Figure 8. Mortality of CD1 mice inoculated with MSSA isolate, HR78 or CSA-1

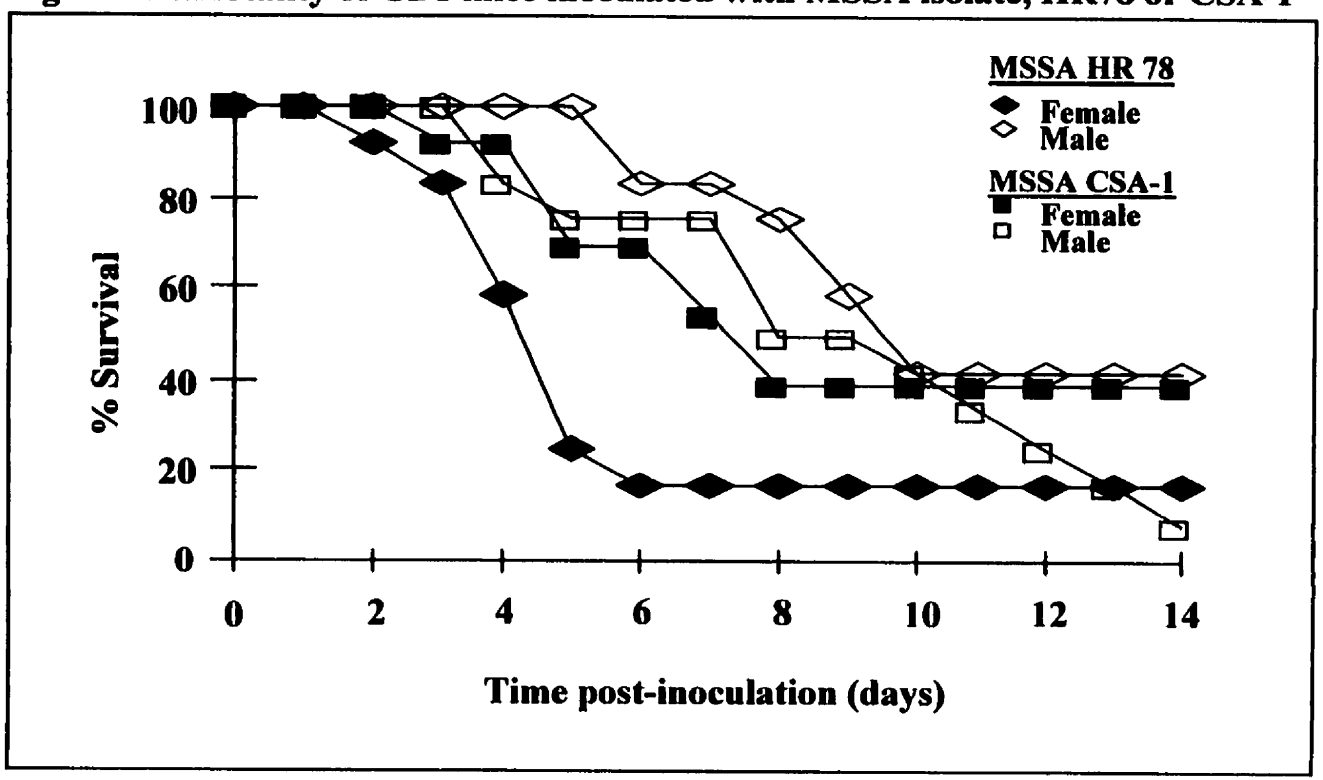
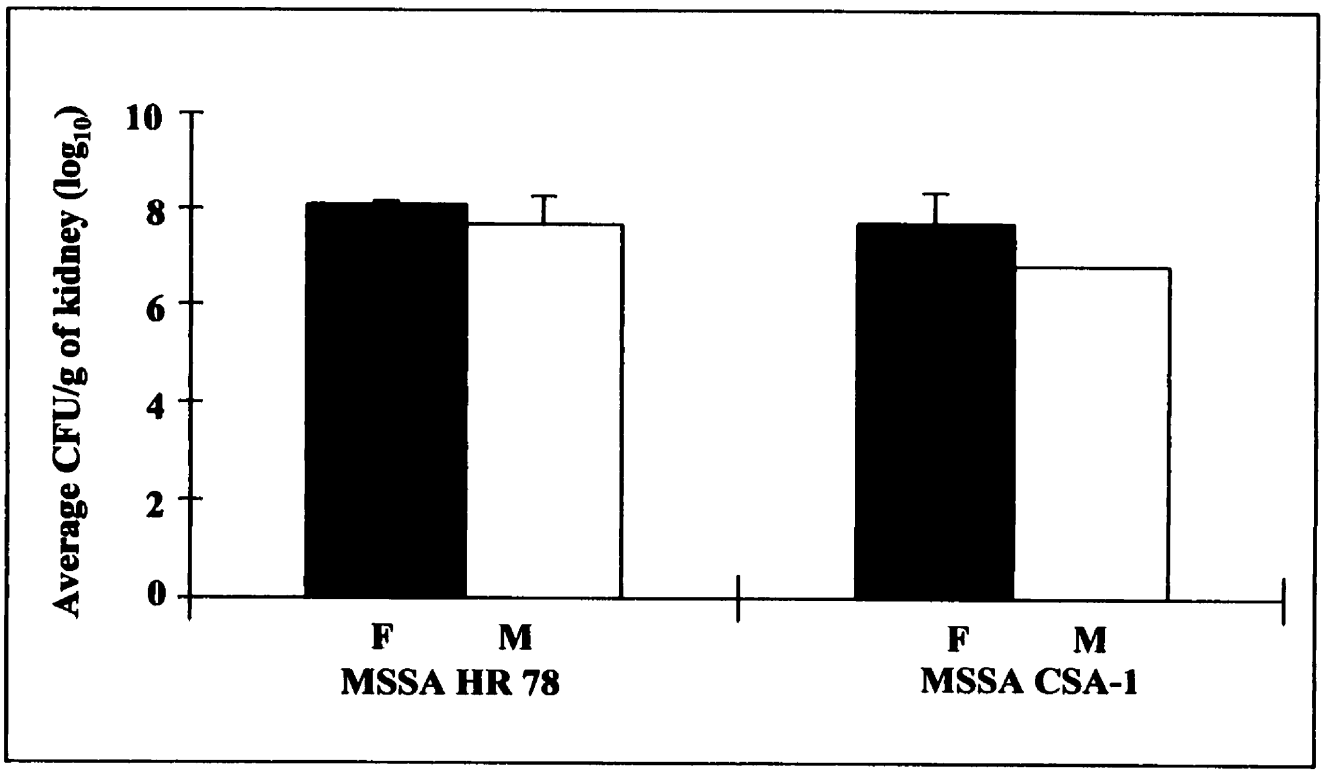


Figure 9. Colonization of the kidneys of male and female CD1 mice surviving to 14 days post-inoculation with MSSA

Colonization of the kidneys of male and female CD1 mice surviving 14 days post-inoculation with MSSA HR78 or MSSA CSA-1. Kidneys were harvested and analyzed for bacterial counts. Data are $\log_{10}$ transformed means where applicable and standard deviation of the means.

Figure 9. Colonization of the kidneys of male and female CD1 mice surviving to 14 days post-inoculation with MSSA



animals. MRSA 456 was arbitrarily chosen for the inoculum as both MRSA isolates previously displayed a gender difference regarding bacterial susceptibility.

3.1.6.1. Kidney Colonization

At 30 days post-inoculation, the number of mice colonized with MRSA 456 was determined and the CFU/g of kidney tissue assessed (Fig. 10). Ninety-one percent of female mice exhibited colonization of the kidney, 100% of castrated male mice had kidney colonization and 67% of the male mice inoculated were colonized with MRSA 456. The number of castrated males colonized was significantly higher than colonization of the non-castrated male mice (P of <0.05). Of the colonized mice, the female mice had the highest average CFU/g of tissue, followed by the castrated males, and the male mice had the lowest average CFU/g of kidney tissue. Kidney colonization was significantly different between the male and female CD1 mice (P of <0.05).

3.1.6.2. Joint Colonization

The number of mice with MRSA colonization of the knee was determined and the CFU/g of knee tissue calculated (Fig. 11). Sixty percent of female mice exhibited colonization of the knee, 100% of castrated male mice had knee colonization and 11% of the non-castrated male mice inoculated were colonized with MRSA 456. Colonization numbers consistently decreased from female to castrated male to male mice. The number of male mice colonized with MRSA 456 significantly differed from the castrated male mice and female mice (P of <0.05).

Figure 10. Effect of castration of male CD1 mice on colonization of the kidney by MRSA 456

Female, male and castrated male CD1 mice were inoculated with 10^7 CFU of MRSA 456. At 30 days post-inoculation, the mice were sacrificed and kidneys analyzed for bacterial colonization. Average CFU/g (log_{10} transformed) of kidney tissue of the mice colonized are shown by the bars (mean +/- SD). The number of mice colonized out of the total mice in that experimental group is displayed above the respective bar. *, significant difference (student's t-test) in average CFU/g of kidney tissue between male and female mice at P of <0.05. ≠, significant difference (Fisher's test) in extent of chronic colonization between castrated and non-castrated male mice at P of <0.05.

Figure 10. Effect of castration of male CD1 mice on colonization of the kidney by MRSA 456

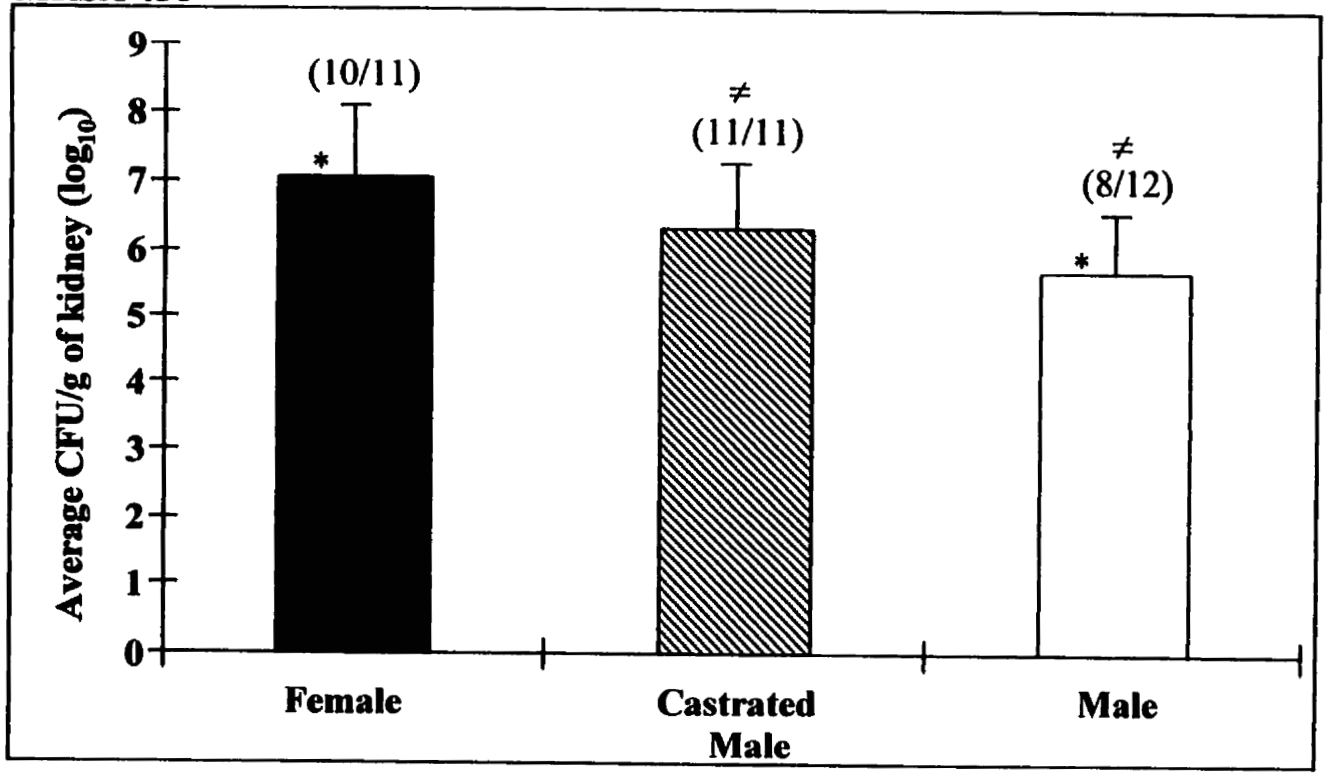
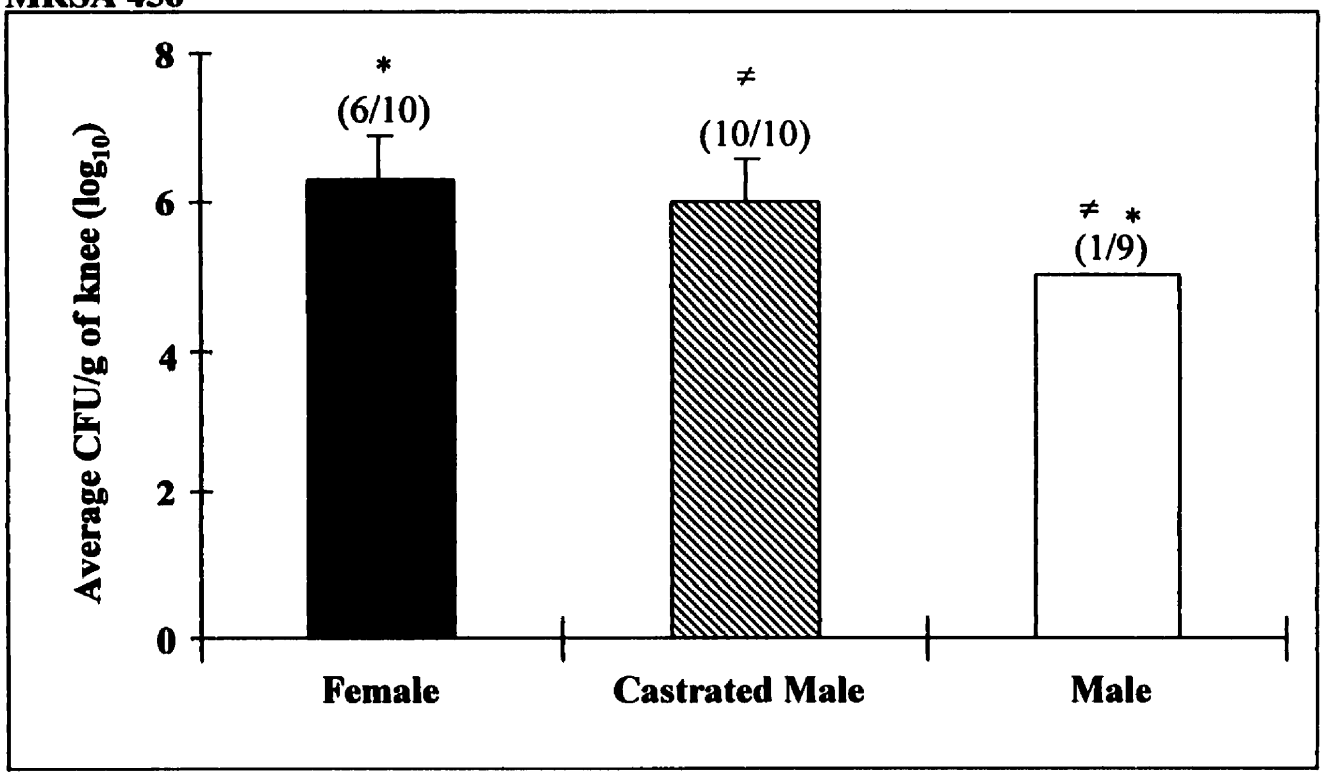


Figure 11. Effect of castration of male CD1 mice on colonization of the knee by MRSA 456

Female, male and castrated male CD1 mice were inoculated with 10^7 CFU of MRSA 456. At 30 days post-inoculation, the mice were sacrificed and knees analyzed for bacterial colonization. Average CFU/g (log_{10} transformed) of knee tissue of the mice colonized are shown by the bars (mean +/- SD). The number of mice colonized out of the total mice in that experimental group is displayed above the respective bar. *, significant difference (Fisher's test) in extent of chronic colonization between male and female mice at P of <0.05. ≠, significant difference (Fisher's test) in extent of chronic colonization between castrated and non-castrated male mice at P of <0.05.

Figure 11. Effect of castration of male CD1 mice on colonization of the knee by MRSA 456



The elbow was also analyzed for joint colonization (Fig. 12). Twenty percent of both female and castrated male mice were colonized with MRSA 456 and none of the male mice displayed elbow colonization. Elbow colonization was similar between the female and castrated male mice. For a given gender, the average CFU/g of colonized elbows was approximately one log factor lower than the average CFU/g of colonized knees. Histological examination of the knee joint revealed microabscesses of bacteria within the muscle and joint capsule (Fig. 13). There was also a high infiltration of PMN's within the joint along with cartilage and tissue destruction (Fig. 14). In response to infection, the host tissue becomes fibrous which is clearly evident within the kidney (Fig. 15).

3.2. UROKINASE DEFICIENT MOUSE MODEL

3.2.1. Organ Colonization

The colonization of different uPA-/- mouse organs was analyzed 48 hours post-inoculation. Organs with bacterial counts greater than 0.1% of the inoculum were considered as colonized, based on the same reasoning given for the CD1 mice. Inoculation of male uPA-/- mice with MRSA resulted in 100% colonization of the mouse kidneys and approximately 50% colonization of the heart (Fig. 16). Minimal colonization of the liver was seen with both MRSA isolates and MRSA 456 was also shown to occasionally colonize the lung. No colonization of the spleen was ever observed. Patterns of colonization for MSSA isolates were similar to those following MRSA inoculation, with the kidney again being the organ predominantly colonized (data not shown).

Figure 12. Effect of castration of male CD1 mice on colonization of the elbow by MRSA 456

Female, male and castrated male CD1 mice were inoculated with 10^7 CFU of MRSA 456. At 30 days post-inoculation, the mice were sacrificed and elbows analyzed for bacterial colonization. Average CFU/g ($\log_{10}$ transformed) of elbow tissue of the mice colonized are shown by the bars. The number of mice colonized out of the total mice is displayed above the respective bar.

Figure 12. Effect of castration of male CD1 mice on colonization of the elbow by MRSA 456

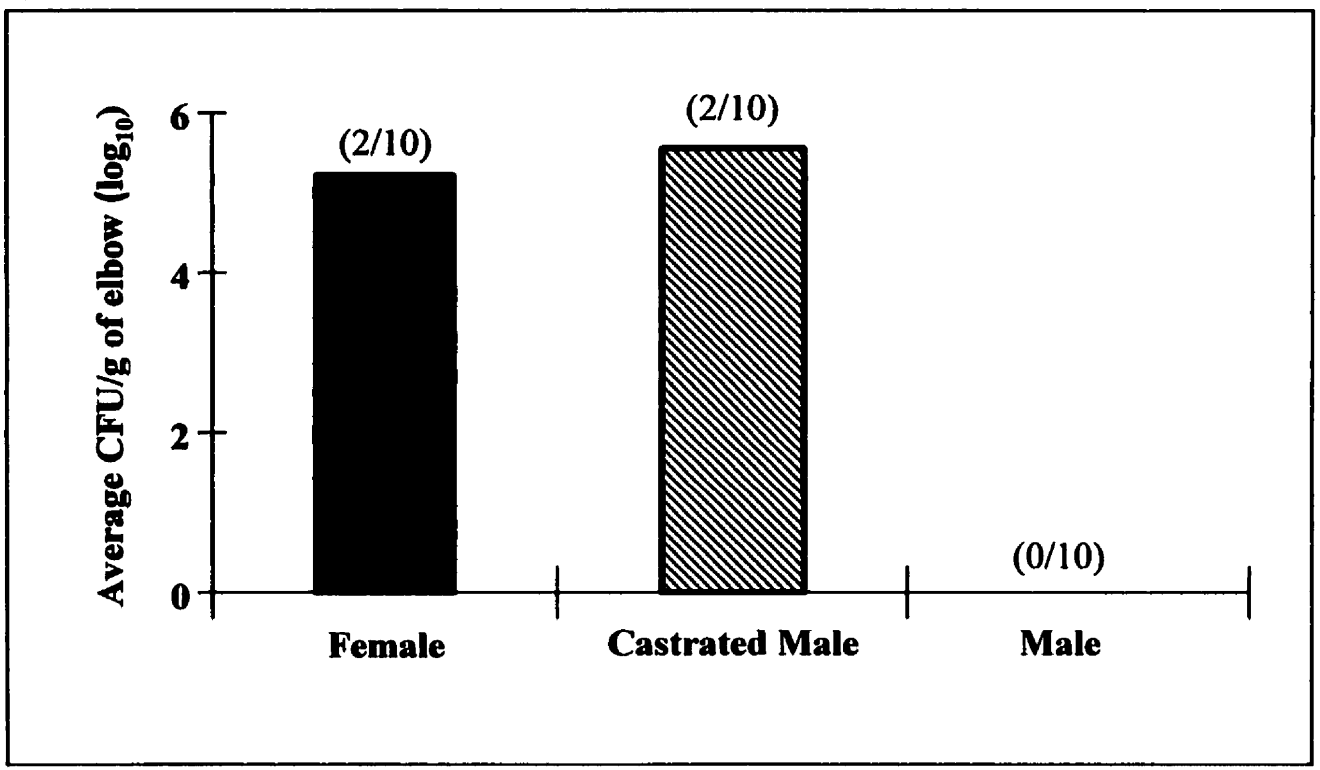


Figure 13. Histology of bacterial colonization of CD1 mouse knee

CD1 mice were inoculated with MRSA 456. One month post-inoculation the knee was harvested and processed for embedding in MMA and staining with Lee's Methylene Blue. A) Normal mouse knee showing cell structure of healthy tissue. B) Infected CD1 mouse knee displaying bacterial microabscesses and overall disarray of tissue structure. Magnification 100X under oil immersion.

Figure 13. Histology of bacterial colonization of CD1 mouse knee

A)

B)

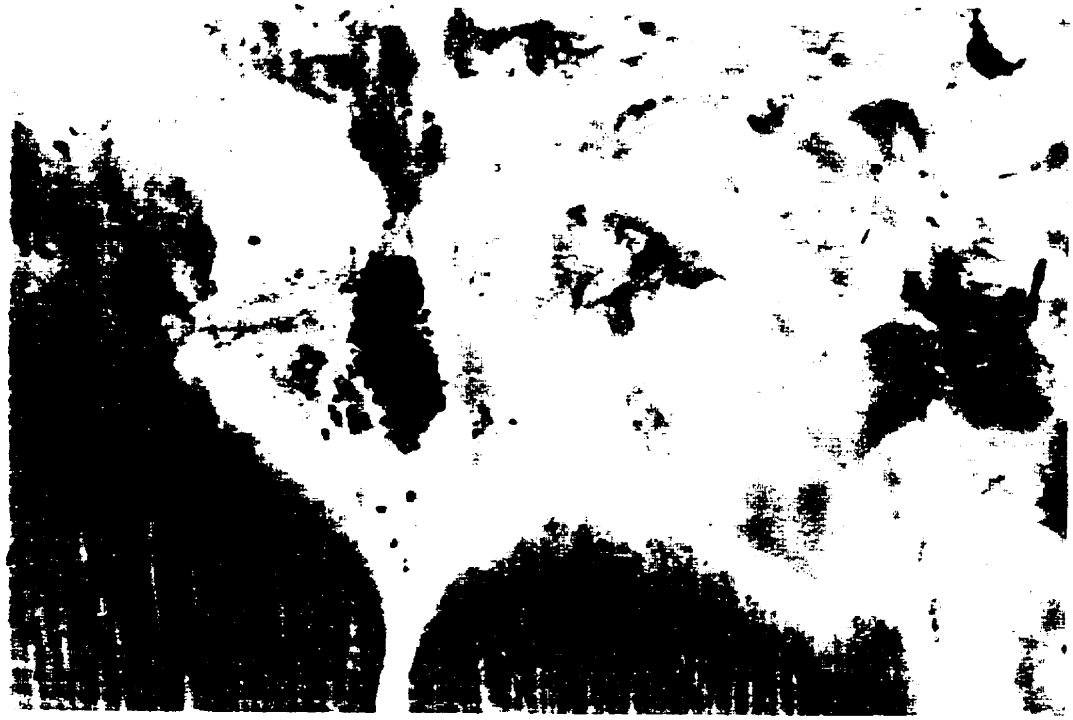
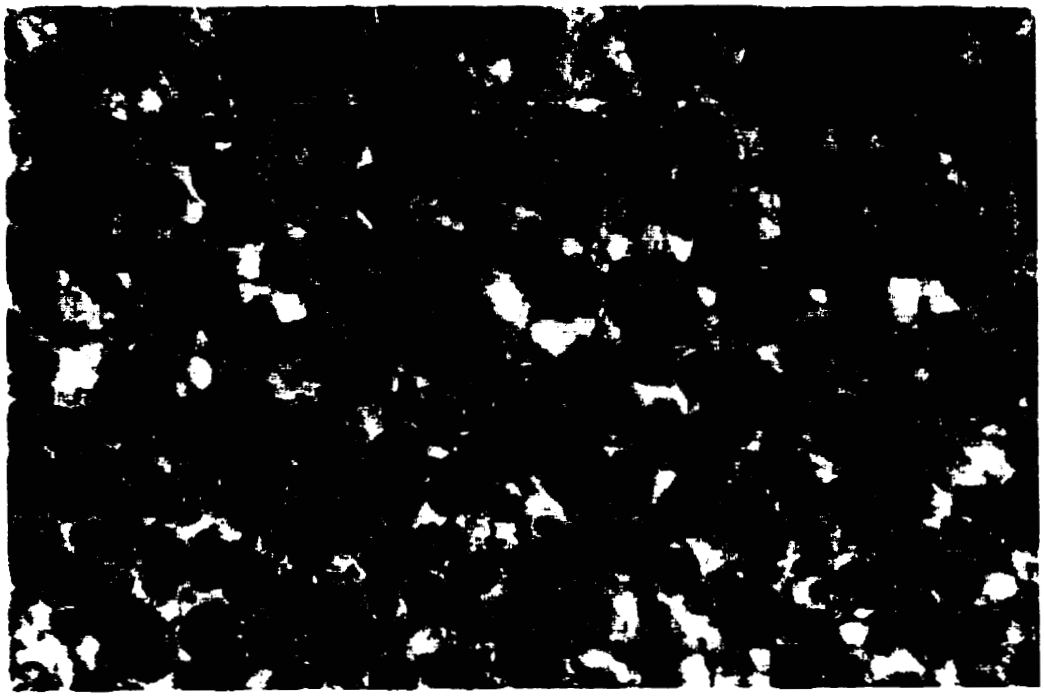


Figure 14. Histology and tissue destruction evident in the knee of CD1 mice inoculated with MRSA

CD1 mice were inoculated with MRSA 456. One month post-inoculation the knee was harvested and processed for embedding in MMA and staining with Lee's Methylene Blue. A) Infiltration of host PMN cells into the knee joint. Magnification 100X under oil immersion. B) Tissue and cartilage destruction within the knee joint. Magnification 40X.

Figure 14. Histology and tissue destruction evident in the knee of CD1 mice inoculated with MRSA

A)



B)

Figure 15. Histology of the knee from CD1 mice inoculated with MRSA

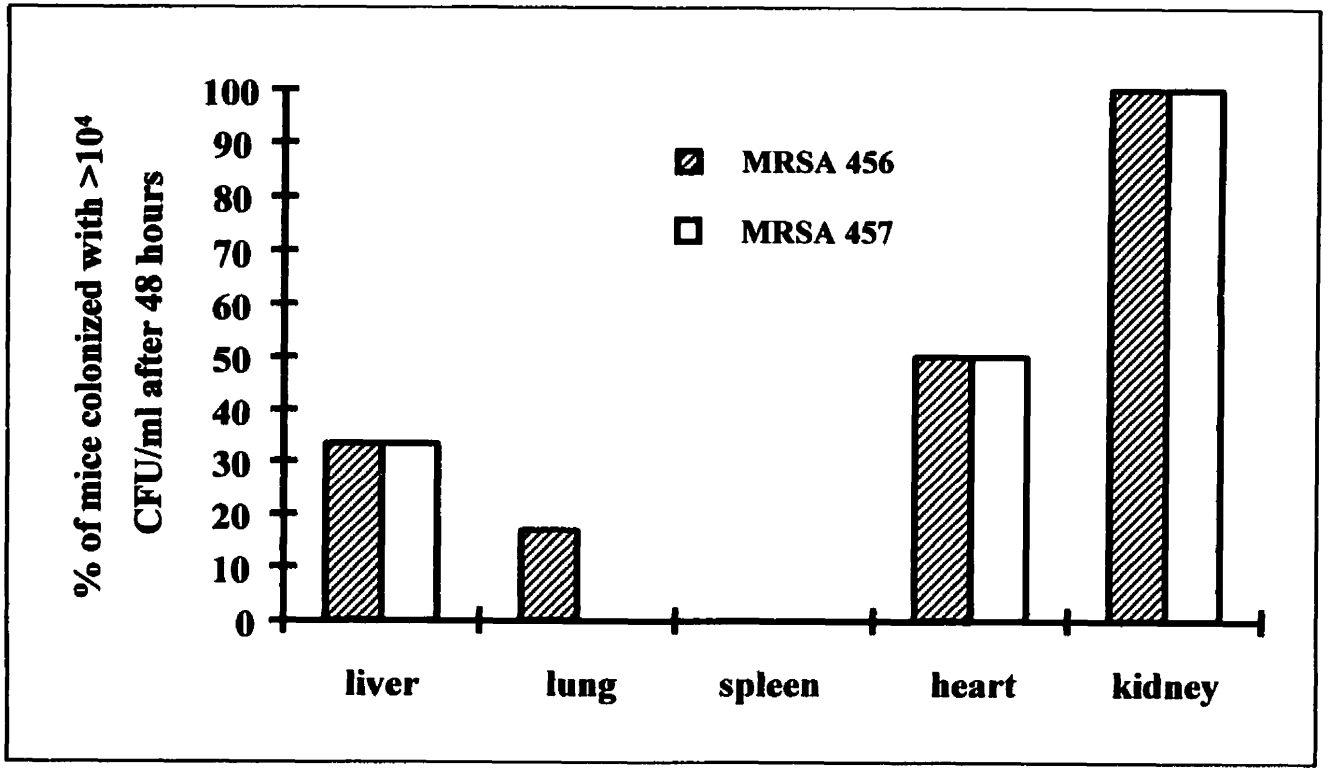
CD1 mice were inoculated with MRSA 456. One month post-inoculation the knee was harvested and processed for embedding in MMA and staining with Lee's Methylene Blue. Evident fibrosis of the knee joint of an infected CD1 mouse. Magnification 40X.

Figure 15. Histology of the knee from CD1 mice inoculated with MRSA

Figure 16. Pattern of colonization of uPA-/- mouse organs by MRSA

uPA-/- mice were inoculated with 10^7 CFU of either MRSA 456 or MRSA 457. After 48 hours, the mice were sacrificed and organs harvested and analyzed for *S. aureus*. Organs with bacterial counts greater than 0.1% of the inoculum were considered as colonized.

Figure 16. Pattern of colonization of uPA-/- mouse organs by MRSA



3.2.2. Inoculum Dosage

Different bacterial inoculum dosages were utilized for inoculations to determine the optimal bacterial dose to induce kidney colonization, with limited mortality, in uPA-/- mice. Male and female mice were inoculated with the appropriate dose of bacteria and monitored up to 2 weeks post-inoculation.

3.2.2.1. Female Survival and Kidney Colonization

Groups of uPA-/- female mice were inoculated with either 10^4, 10^6, 10^7 or 10^8 CFU of MRSA 456. Female mice inoculated with 10^8 CFU of MRSA 456 showed 0% survival (Fig. 17). Inoculation of the mice with 10^7 CFU had a slightly less detrimental effect, with 53% of the mice surviving. At a bacterial dose of 10^6 CFU 80% of the mice survived and at 10^4 CFU of MRSA 456, 83% of the inoculated mice survived up to 14 days post-inoculation. The majority of deaths in all of the inoculum groups occurred by one week post-inoculation (Fig. 17). To examine the colonization of MRSA 456 in the surviving mice, CFU/g of kidney was calculated for these mice (Fig. 18). Moving from the lowest dose of bacteria to the highest, there is an increase in kidney colonization CFU counts and the number of surviving mice colonized in the kidney. There were no survivors from the 10^8 inoculum group to analyze. Female mice inoculated with 10^4 CFU of MRSA 456 had no colonization of the kidney, which was significantly different from both the 10^6 dose with 75% of the surviving mice colonized and the 10^7 dose with 7/7 surviving mice colonized (Fig. 18).

Figure 17. Influence of inoculation dose of MRSA 456 on mortality of female uPA-/- mice

Female mice were inoculated with varying doses of MRSA 456 and monitored up to 14 days post-inoculation. Female mice inoculated with 10^4 CFU of MRSA 456 (n=6), 10^6 CFU of MRSA 456 (n=5), 10^7 CFU of MRSA 456 (n=13), or 10^8 CFU of MRSA 456 (n=9). *, significant difference (Fisher's t-test) in the % survival between mice inoculated with 10^8 CFU and 10^7 CFU at P of <0.05. ≠, significant difference (Fisher's test) in the % survival between mice inoculated with 10^8 CFU and 10^6 CFU at P of <0.05. Φ, significant difference (Fisher's test) in the % survival between mice inoculated with 10^8 CFU and 10^4 CFU at P of <0.05.

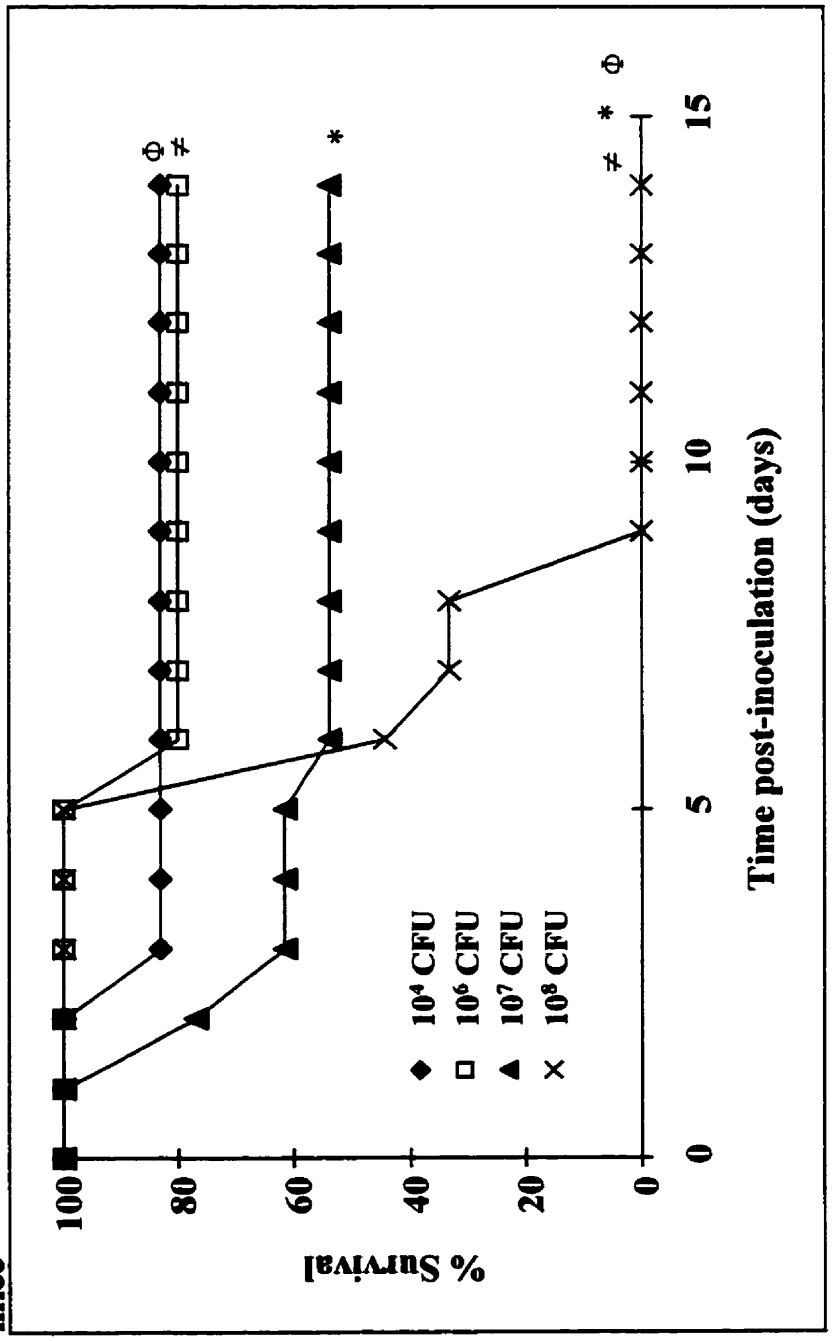
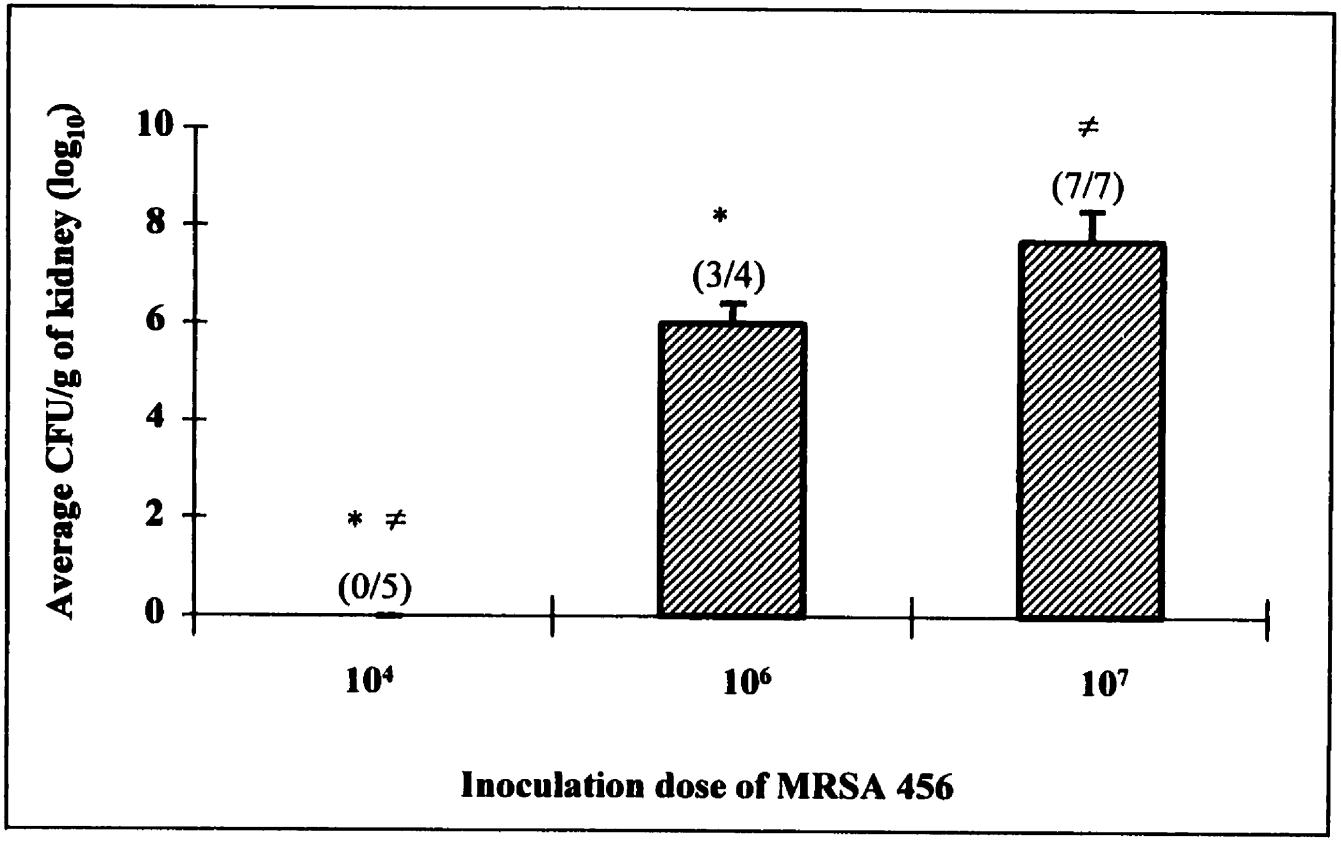


Figure 17. Influence of inoculation dose of MRSA 456 on mortality of female uPA-/- mice

Figure 18. Influence of inoculation dose of MRSA 456 on kidney colonization of female uPA-/- mice

Female uPA-/- mice were inoculated with different doses of MRSA 456. At 14 days post-inoculation, the mice were sacrificed and kidneys assessed for bacterial colonization. There were no survivors inoculated with 10^8 CFU of MRSA 456. Average CFU/g (log_{10} transformed) of kidney tissue of the mice colonized at different inoculation doses are shown by the bars (mean +/- SD). The number of mice colonized out of the total mice/group is displayed above the respective bar. *, significant difference (Fisher's t-test) in the extent of kidney colonization between mice inoculated with 10^4 CFU and 10^6 CFU at P of <0.05. ≠, significant difference (Fisher's test) in extent of chronic colonization between mice inoculated with 10^4 CFU and 10^7 CFU at P of <0.05.

Figure 18. Influence of inoculation dose of MRSA 456 on kidney colonization of female uPA-/- mice



3.2.2.2. Male Survival and Kidney Colonization

Groups of uPA-/- male mice were inoculated with either 10^3, 10^5, 10^6 or 10^7 CFU of MRSA 456. Male mice inoculated with 10^3 CFU of MRSA 456 had a 100% survival rate (Fig. 19). With an increase in inoculum dosage the survival rate decreased. Mice inoculated with 10^5 CFU of MRSA 456 had a survival rate of 85%, mice with a 10^6 CFU inoculation had a 83% survival rate and mice with a 10^7 CFU inoculation had a 27% survival rate (Fig. 19). The majority of deaths occurred prior to 5 days post-inoculation. To examine the colonization of MRSA 456 in the surviving mice, CFU/g of kidney tissue was calculated for these mice (Fig. 20). As the bacterial inoculum increases so did the number of surviving mice colonized and the CFU/g of kidney tissue. Male mice inoculated with 10^3 CFU of MRSA 456 had no colonization of the kidneys and this was significantly different from mice inoculated with 10^6 and 10^7 CFU of MRSA 456 which both showed over 80% of kidneys colonized in surviving mice (P of <0.05). Surviving male mice inoculated with 10^5 CFU of MRSA 456 had a 50% colonization rate in the kidney.

3.2.3. MRSA Colonization of the Kidney

Kidney colonization was analyzed to determine if MRSA 456 and MRSA 457 behaved differently *in vivo* with respect to growth. MRSA 456 and MRSA 457 exhibited similar patterns of colonization in the female CD1 mice. With an initial inoculation dose of 10^7 CFU/mouse, over 14 days post-inoculation the CFU/g of kidney tissue increased approximately one log value for both MRSA 456 and MRSA 457 (Fig. 21). MRSA 457

Figure 19. Influence of inoculation dose of MRSA 456 on mortality of male uPA-/- mice

Groups of male mice were inoculated with either 10^3 (n=5), 10^5 (n=7), 10^6 (n=6), or 10^7 (n=22) CFU of MRSA 456. The mice were monitored up to 14 days post-inoculation, when the survivors were sacrificed. *, significant difference (Fisher's t-test) in the % survival between mice inoculated with 10^7 CFU and 10^6 CFU at P of <0.05. ≠, significant difference (Fisher's test) in the % survival between mice inoculated with 10^7 CFU and 10^5 CFU at P of <0.05. Φ, significant difference (Fisher's test) in the % survival between mice inoculated with 10^7 CFU and 10^3 CFU at P of <0.05.

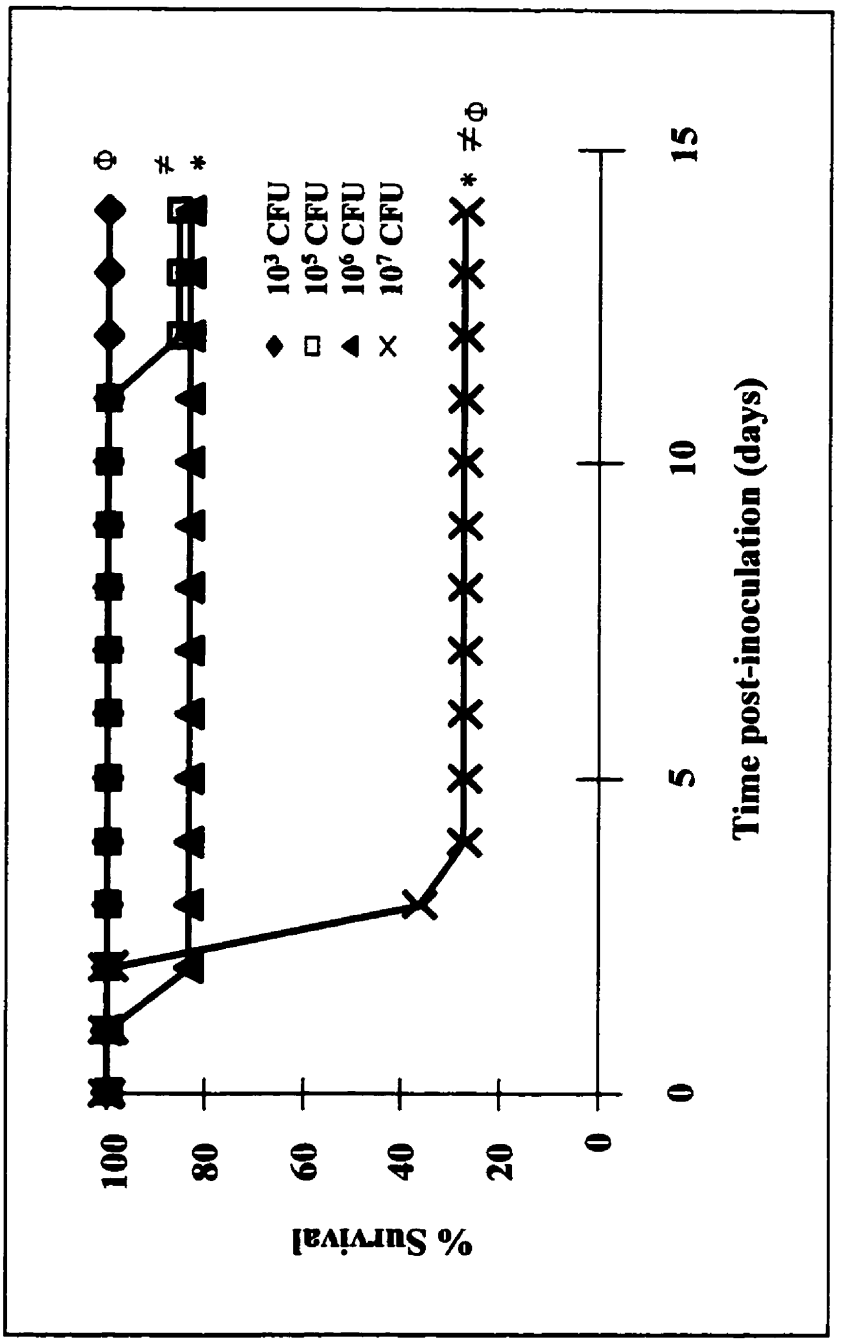


Figure 19. Influence of inoculation dose of MRSA 456 on mortality of male uPA-/- mice

Figure 20. Influence of inoculation dose of MRSA 456 on kidney colonization of male uPA-/- mice

Male uPA-/- mice were inoculated with different doses of MRSA 456. At 14 days post-inoculation, the mice were sacrificed and kidneys studied for bacterial colonization. Average CFU/g (log_{10} transformed) of kidney tissue of the mice colonized at different inoculation doses are shown by the bars (mean +/- SD). The number of mice colonized out of the total mice in that experimental group is displayed above the respective bar. *, significant difference (Fisher's t-test) in the extent of kidney colonization between mice inoculated with 10^3 CFU and 10^6 CFU at P of <0.05. ≠, significant difference (Fisher's test) in extent of chronic colonization between mice inoculated with 10^3 CFU and 10^7 CFU at P of <0.05.

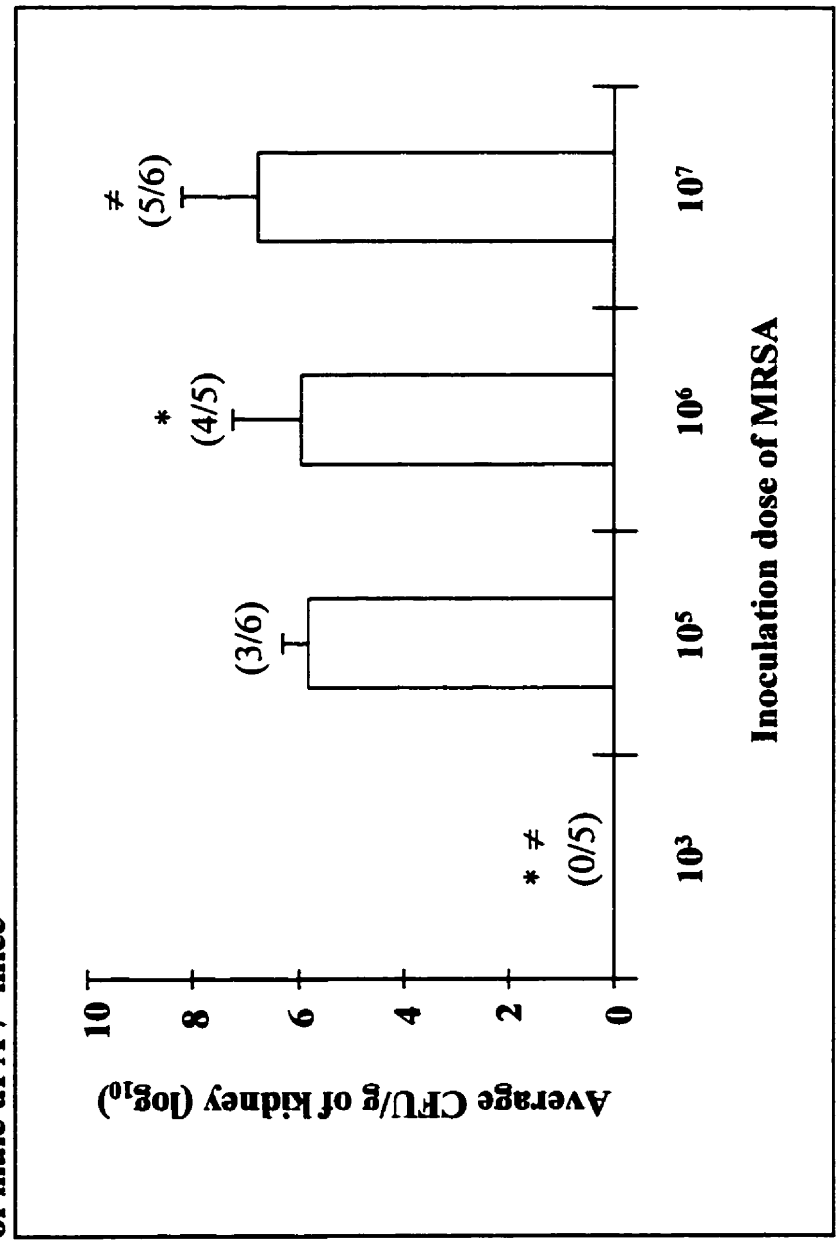
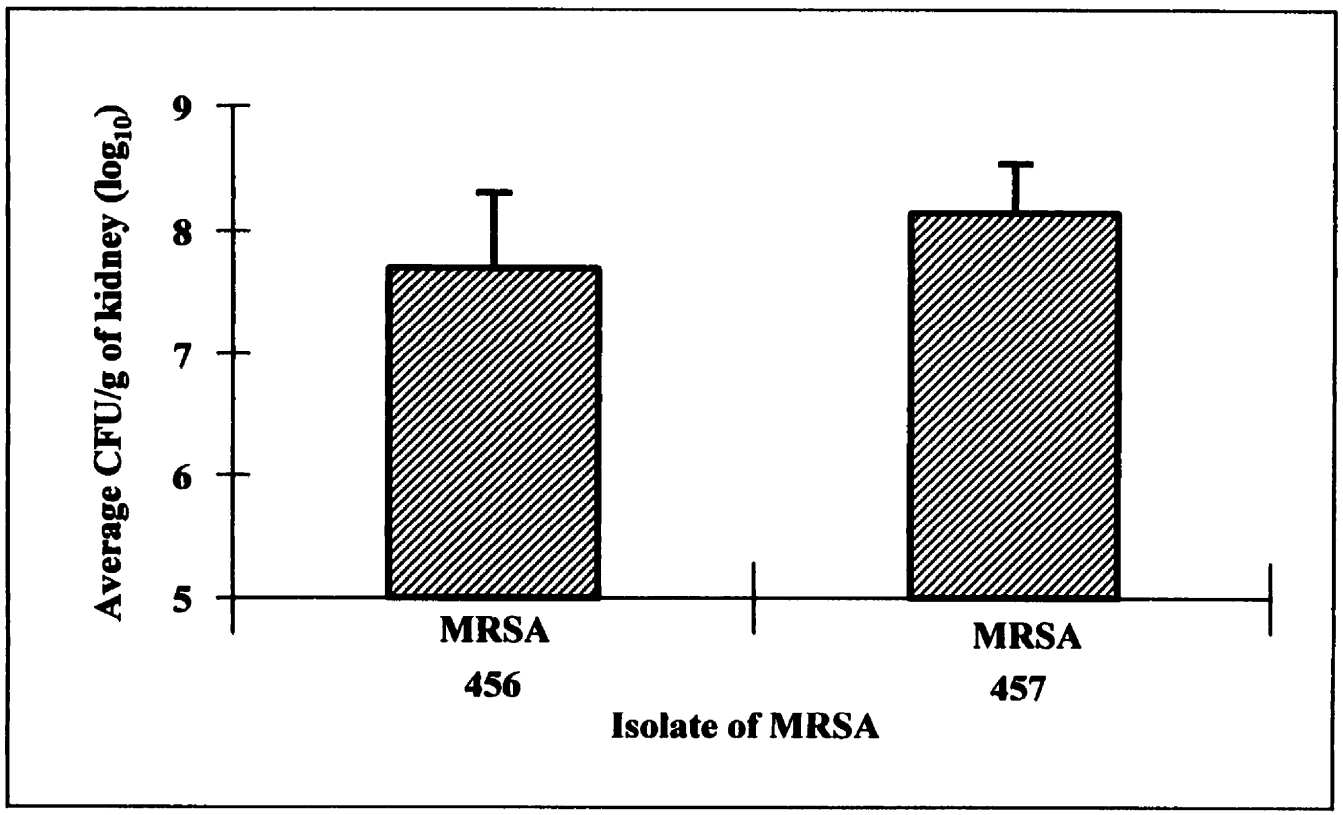


Figure 20. Influence of inoculation dose of MRSA 456 on kidney colonization of male uPA-/- mice

Figure 21. Colonization of the kidney at two weeks post-inoculation in uPA-/- female mice inoculated with MRSA

Female uPA-/- mice were inoculated with the indicated MRSA isolate (456 or 457) and sacrificed at 14 days post-inoculation. The kidneys were harvested and processed for quantitative bacteriology. Data are presented as $\log_{10}$ transformed means +/- standard deviation of the means where n=6.

Figure 21. Colonization of the kidney at two weeks post-inocualtion in uPA-/- female mice inoculated with MRSA



did show a slightly higher levels of colonization than MRSA 456, but the differences were not significant. The CD1 male mice showed greater colonization by MRSA 457 than MRSA 456 up to 14 days post-inoculation, but values were never significantly different (Fig. 22). At 14 days post-inoculation, MRSA 457 inoculated mice had an average colonization of 1.5 logs higher than the inoculation dose and MRSA 456 inoculated mice had an average colonization of approximately one log lower than the initial inoculation dose. Histological examination showed the presence of bacteria in the glomeruli and tubules along with infiltration of host inflammatory cells in some infected areas (Fig. 23). Evident bacterial microabscesses were formed within the kidneys resulting in large amounts of tissue damage (Fig. 24) There did not appear to be any obvious difference in histology between mice inoculated with MRSA 456 or MRSA 457.

3.2.4. MRSA Survival Rates and Kidney Colonization

Survival of MRSA inoculated mice was assessed to compare the *in vivo* behavior of MRSA 456 and MRSA 457 and to determine if a difference in susceptibility between male and female mice to these MRSA isolates exists. Male and female mice inoculated with either isolate of MRSA showed a gradual decrease in survival up to 14 days post-inoculation (Fig. 25). All groups of mice inoculated had a final survival rate less than 70%. Seven out of thirteen female mice inoculated with MRSA 456 survived, 6/12 female mice inoculated with MRSA 457 survived and 7/11 male mice inoculated with MRSA 457 survived. Male mice inoculated with MRSA 456 had the lowest survival rate with 2/7 mice surviving at 14 days post-inoculation (Fig. 25). Assessment of

Figure 22. Colonization of the kidney up to two weeks post-inoculation in uPA-/- male mice inoculated with MRSA

Groups of uPA-/- male mice were inoculated with 10^7 CFU of either MRSA 456 or MRSA 457 and then sacrificed at different time points post-inoculation. Kidneys were harvested and bacterial counts determined and expressed as CFU/g of kidney. Data are $\log_{10}$ transformed averages and standard deviation of the means where n=10.

Figure 22. Colonization of the kidney two weeks post-inocualtion in uPA-/- male mice inoculated with MRSA

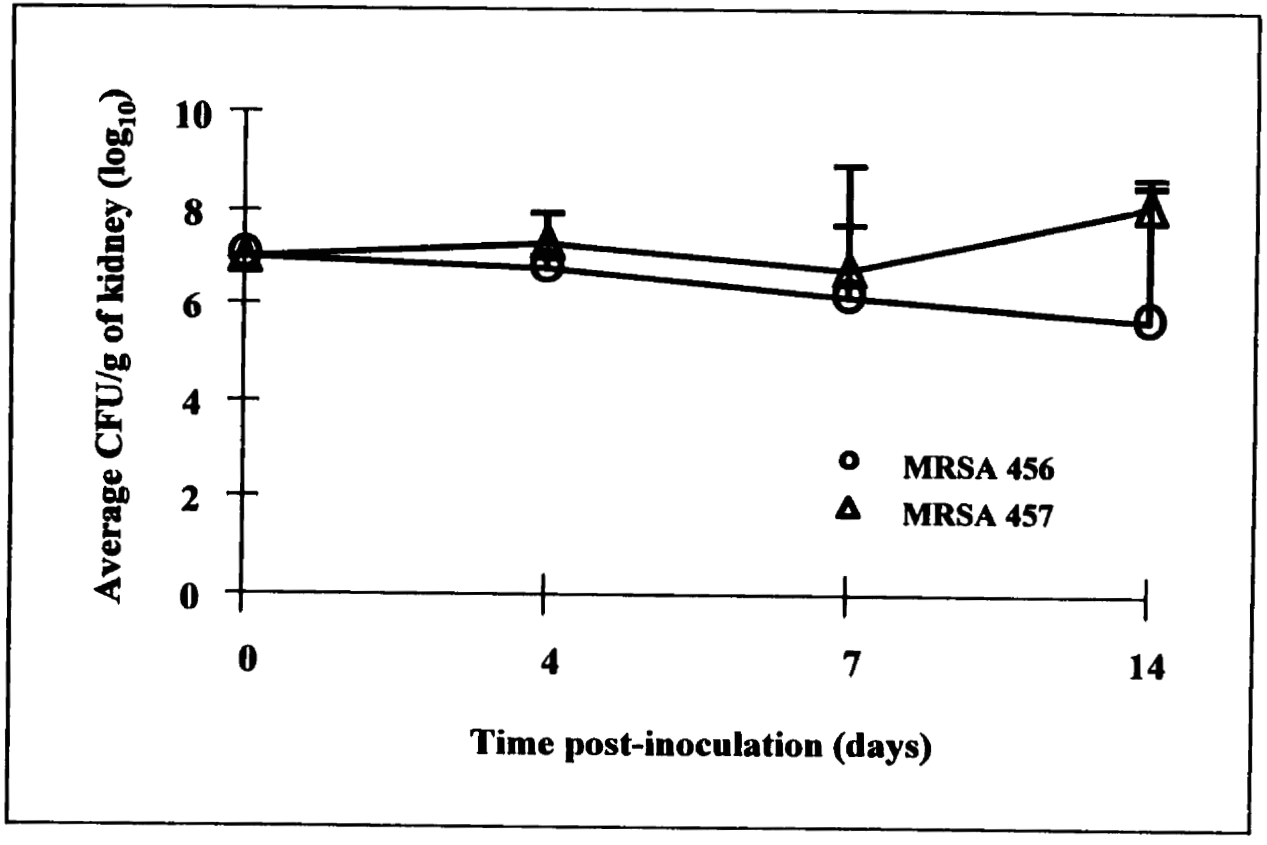
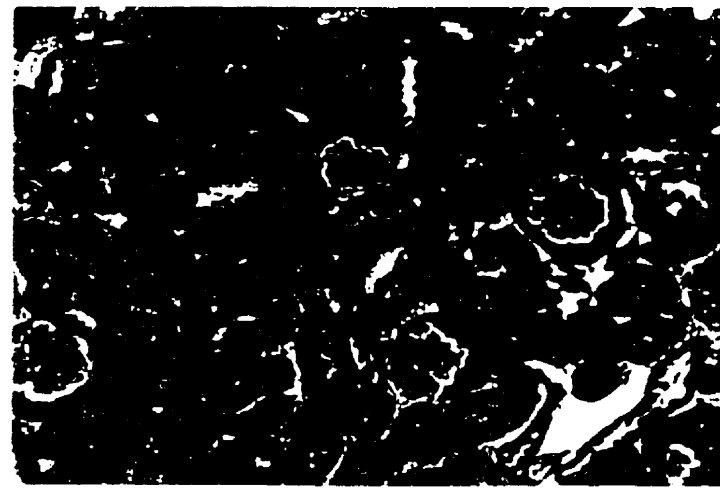


Figure 23. Bacteria in the glomeruli and tubules of the kidneys from uPA-/- mice inoculated with MRSA

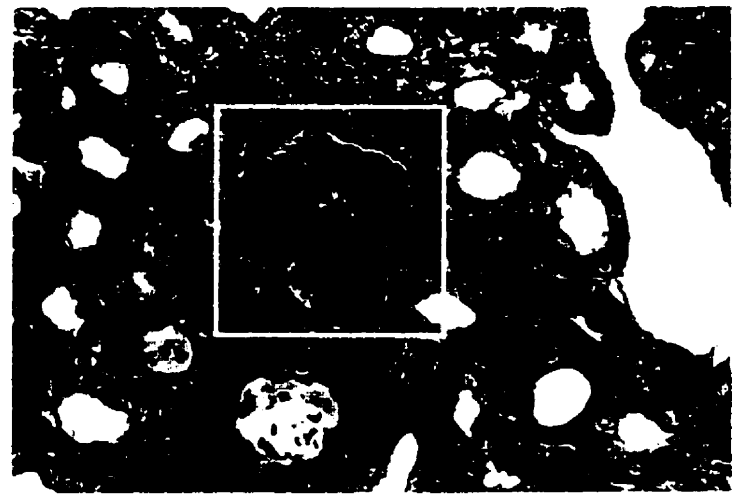
uPA-/- mice were inoculated with MRSA and sacrificed 14 days post-inoculation. At the time of sacrifice, the kidneys were removed and prepared for histological processing, embedded in MMA, and stained with Lee's Methylene Blue. A) Normal mouse kidney showing healthy tubules and glomeruli. Magnification 25X. B) Kidney from an infected uPA-/- mouse showing bacterial microabscesses in the glomeruli and tubules. Magnification 40X. C) Glomeruli of the kidney with bacterial colonization. Magnification 100X under oil immersion.

Figure 23. Bacteria in the glomeruli and tubules of the kidneys from uPA-/-mice inoculated with MRSA

A)



B)



C)

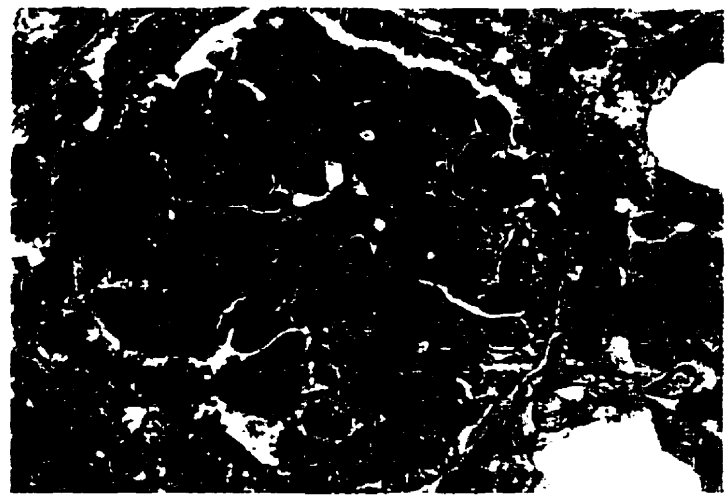


Figure 24. Bacterial microabscesses in the kidneys of uPA-/- mice inoculated with MRSA

uPA-/- mice were inoculated with MRSA and sacrificed 14 days post-inoculation. At the time of sacrifice the kidneys were removed and prepared for histological processing, embedded in MMA, and stained with Lee's Methylene Blue. A) Bacterial microabscesses and tissue destruction of the mouse kidney. Tissue fibrosis is also evident. Magnification 40X. B) Bacterial microabscess. Magnification 100X under oil immersion.

Figure 24. Bacterial microabscesses in the kidneys of uPA-/- mice inoculated with MRSA

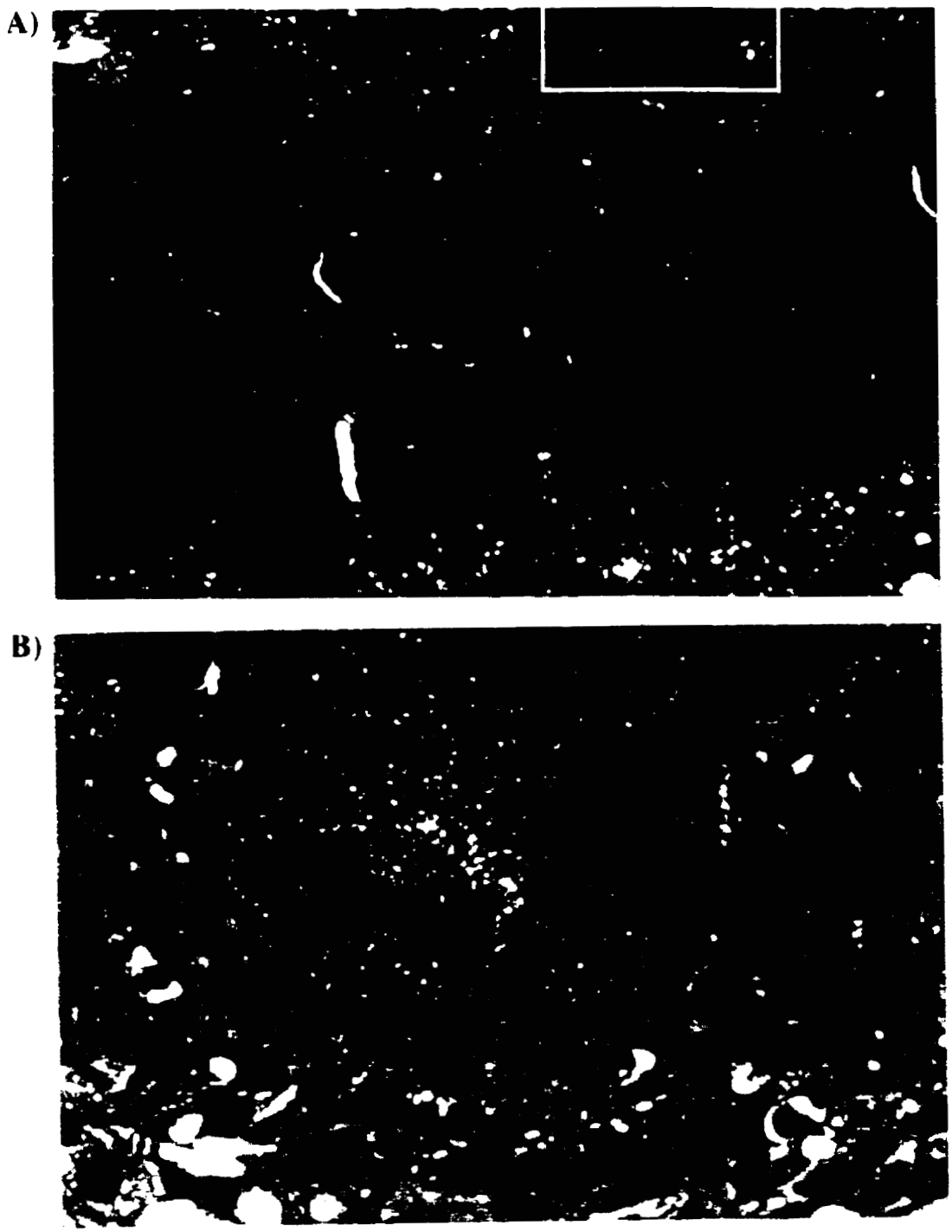
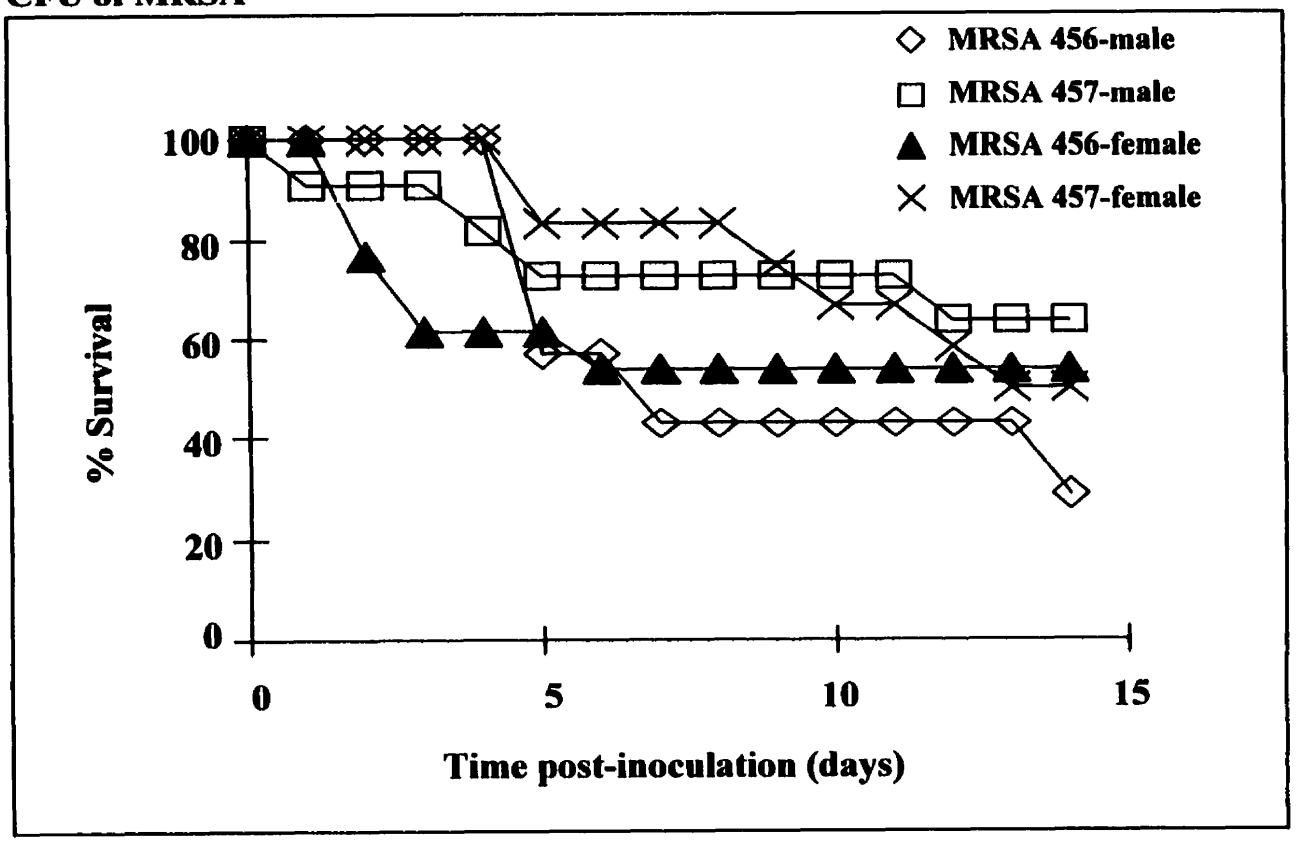


Figure 25. Mortality according to gender of uPA-/- mice inoculated with 10^7 CFU of MRSA

Female and male uPA-/- mice were inoculated with 10^7 CFU of MRSA and monitored up to 14 days post-inoculation. Male mice inoculated with MRSA 456 n=7, male mice inoculated with MRSA 457 n=11, female mice inoculated with MRSA 456 n=13 and female mice inoculated with MRSA 457 n=12.

Figure 25. Mortality according to gender in uPA-/- mice inoculated with 10^7 CFU of MRSA



bacterial counts in the kidneys of surviving uPA-/- mice indicated that female mice displayed similar levels of colonization with either isolate of MRSA (Fig. 26). Male mice however, had significantly greater colonization with MRSA 456 than MRSA 457 (P of <0.05). Inoculation with MRSA 457 displayed a gender difference in bacterial colonization levels. Female mice had significantly higher levels of kidney colonization than corresponding male mice (Fig. 26) (P of <0.05). Of the surviving mice inoculated with MRSA 456, 100% showed colonization of the kidney. Surviving male mice inoculated with MRSA 457 showed 4/7 having kidney colonization and 5/6 female mice inoculated with MRSA 457 displayed colonization of the kidney. Following inoculation of mice with MRSA isolates, there appeared to be a gender difference in mouse colonization with MRSA 457, but the two isolates of MRSA exhibited similar levels of mortality. Histology sections of the mice kidneys, showed infiltration of polymorphonuclear cells (PMNs) and microcolonies of bacteria throughout the kidneys. There was no overt differences in the kidney histological preparations of mice inoculated with MRSA 456 compared to mice inoculated with MRSA 457.

3.2.5. MSSA Survival Rates and Kidney Colonization

Two MSSA isolates, HR78 and CSA-1, were used to compare the *in vivo* virulence of MRSA and MSSA isolates in the uPA-/- mice. The effect of gender on susceptibility to MSSA infection as well as the influence of *in vitro* UK responsiveness on *in vivo* growth were evaluated. MSSA inoculation proved to be more lethal than MRSA to both male and female mice (Fig. 27). Both male and female mice inoculated

Figure 26. Colonization of uPA-/- kidneys of mice surviving two weeks post-inoculation with MRSA

Colonization of the kidneys of male (M, ☐) and female (F, ■) uPA-/- mice surviving inoculation with MRSA 456 or MRSA 457. Kidneys were harvested and analyzed for bacterial counts at 14 days post-inoculation. Data presented are $\log_{10}$ transformed means +/- standard deviation of the mean. The number of mice colonized out of the total mice in that experimental group is displayed above the respective bar. *, significant difference between male and female mice inoculated with MRSA 457 at P of <0.05. ≠, significant difference between male mice inoculated with MRSA 457 versus MRSA 456 at P of <0.05.

Figure 26. Colonization of uPA-/- kidneys of mice surviving two weeks post-inoculation with MRSA

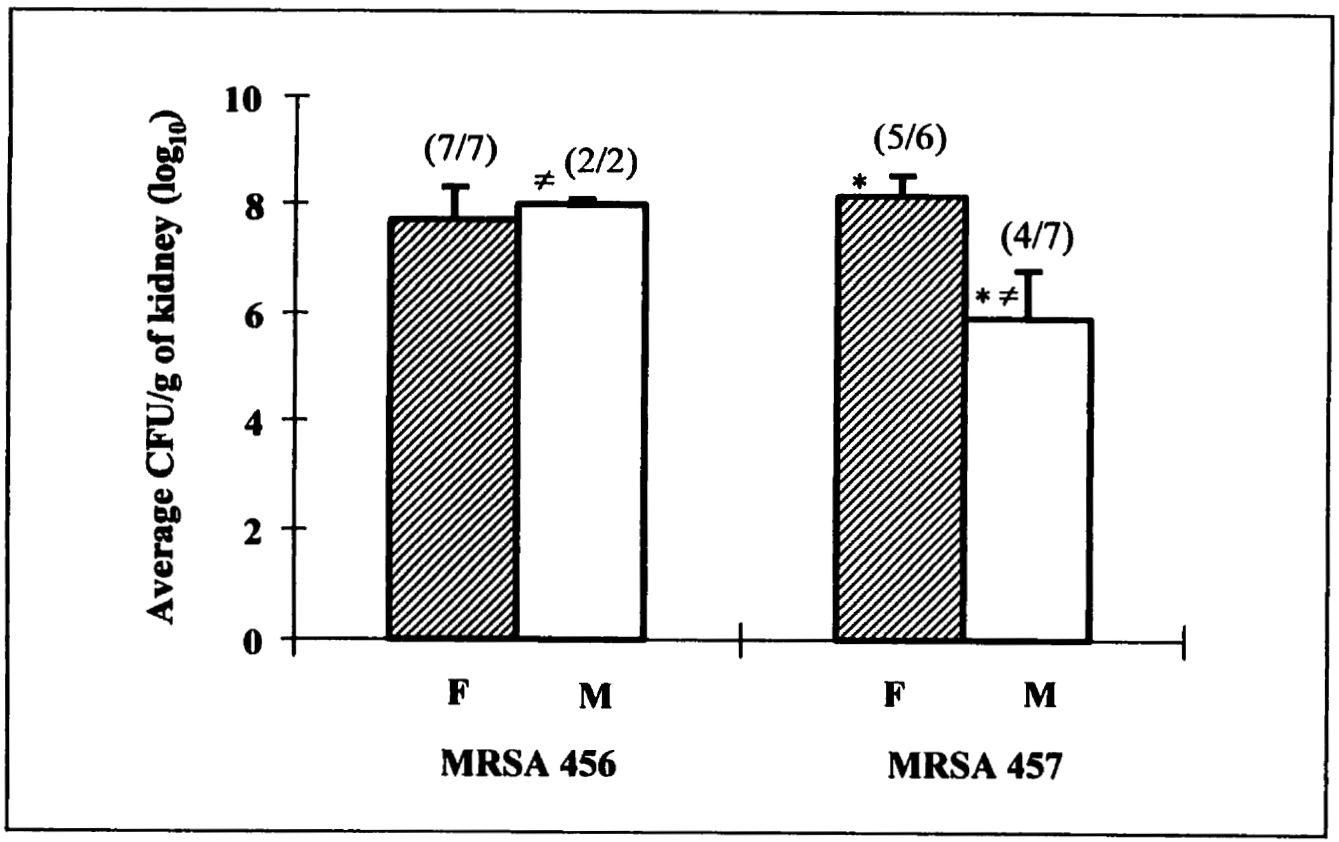
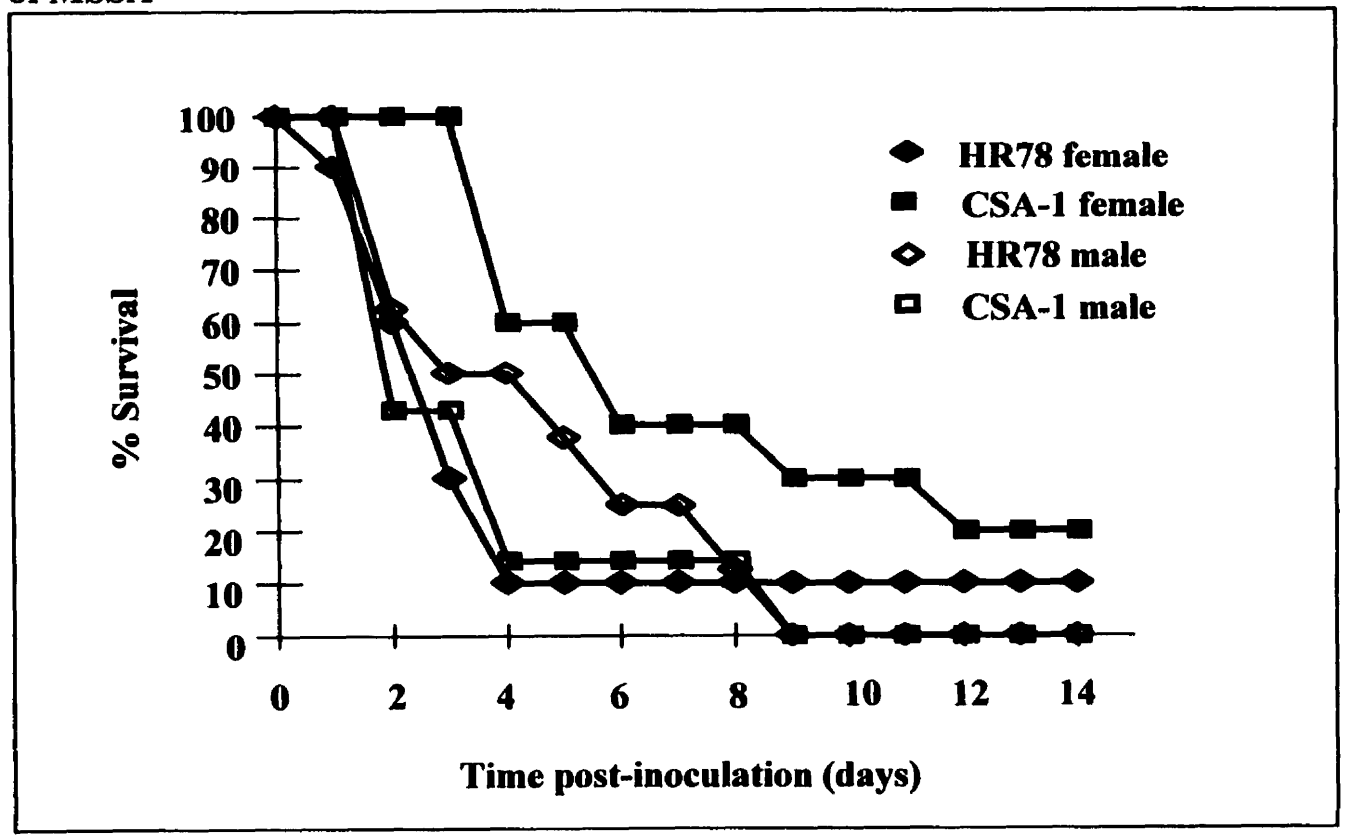


Figure 27. Mortality according to gender of uPA-/- mice inoculated with 10^7 CFU of MSSA

Groups of male (n=8) and female (n=10) uPA-/- mice were inoculated with MSSA isolates and observed for 14 days post-inoculation. Deaths were recorded daily.

Figure 27. Mortality according to gender in uPA-/- mice inoculated with 10^7 CFU of MSSA



with either isolate of MSSA showed drastic declines in survival by one week post-inoculation. By 9 days post-inoculation 0% of the initial male mice inoculated were alive. Female mice inoculated with MSSA HR78 showed 1/10 survived up to 14 days post-inoculation and with MSSA CSA-1 2/10 survived up to 14 days post-inoculation (Fig. 27). To examine the influence of MSSA isolates in the surviving mice, CFU/g of kidney tissue was calculated for these mice (Fig. 28). Female mice surviving to day 14 exhibited similar levels of kidney colonization and there was no evident difference between colonization by HR78 and CSA-1. Histological examination displayed no evident difference between mouse kidneys inoculated with MSSA HR78 and MSSA CSA-1 (data not shown).

3.2.6. Joint Colonization

The knee and elbow joints of female mice were analyzed to determine if MRSA 456 and MRSA 457 behave differently *in vivo* with regard to joint colonization. The number of mice with MRSA colonization of the knee and/or elbow was determined and the CFU/g of tissue calculated (Fig. 29). Colonization by the two MRSA isolates displayed similar results for both the knee and the elbow. At 14 days post-inoculation, joint colonization was approximately 10^7 CFU/g of tissue.

Figure 28. Colonization of uPA-/- kidneys of mice surviving two weeks post-inoculation with MSSA

Colonization of the kidneys of female uPA-/- mice surviving 14 days post-inoculation with 10^7 CFU of either MSSA HR78 or MSSA CSA-1. Kidneys were harvested and analyzed for bacterial counts. Data are $\log_{10}$ transformed values.

Figure 28. Colonization of uPA-/- kidneys of mice surviving two weeks post-inoculation with MSSA

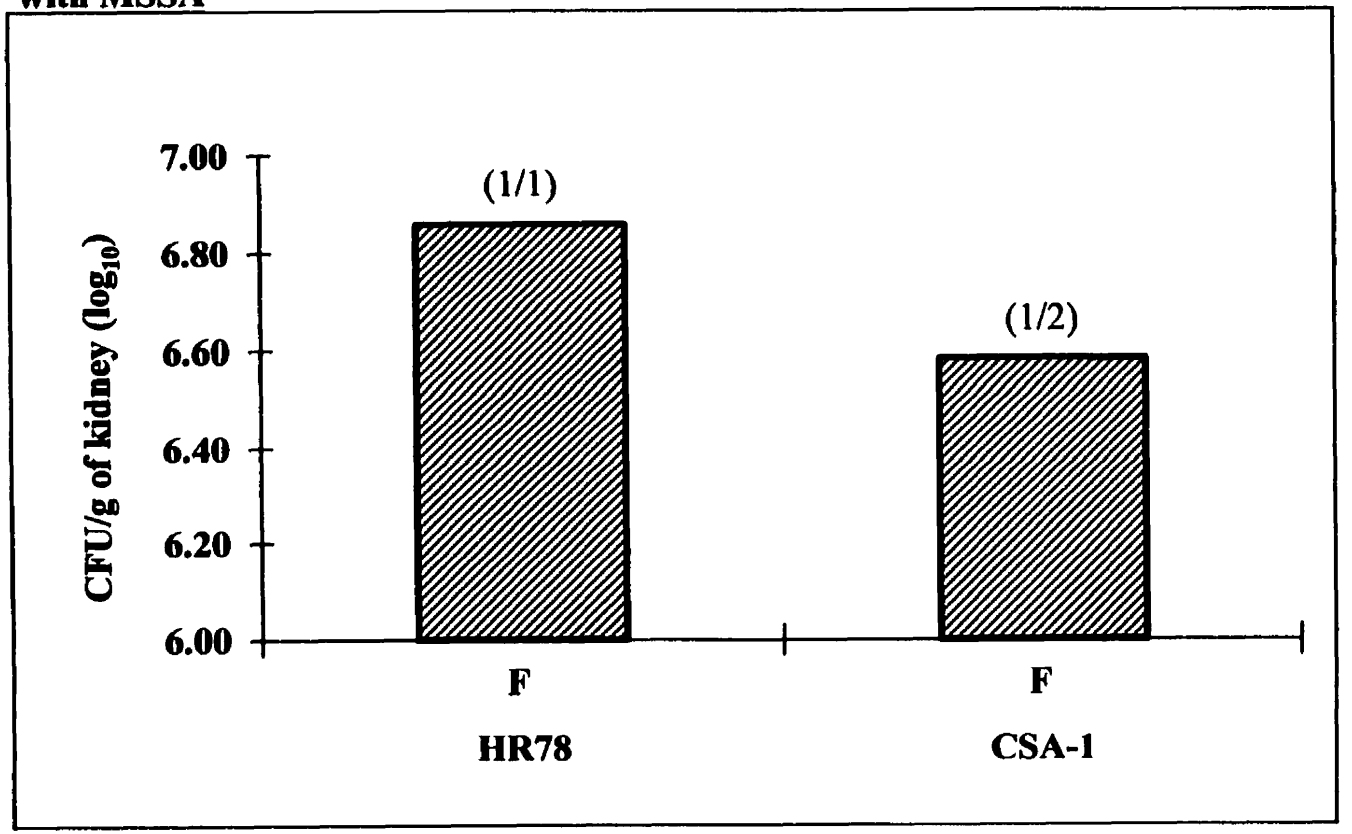


Figure 29. Joint colonization of female uPA-/- mice inoculated with MRSA

Female uPA-/- mice were inoculated with 10^7 CFU of MRSA 456 or MRSA 457. At 14 days post-inoculation, the mice were sacrificed and knees and elbows assessed for bacterial colonization. Average CFU/g ($\log_{10}$ transformed) of joint tissue of the mice colonized are shown by the bars (mean +/- SD). All groups n=5 except the elbow colonization with MRSA 457 where n=1.

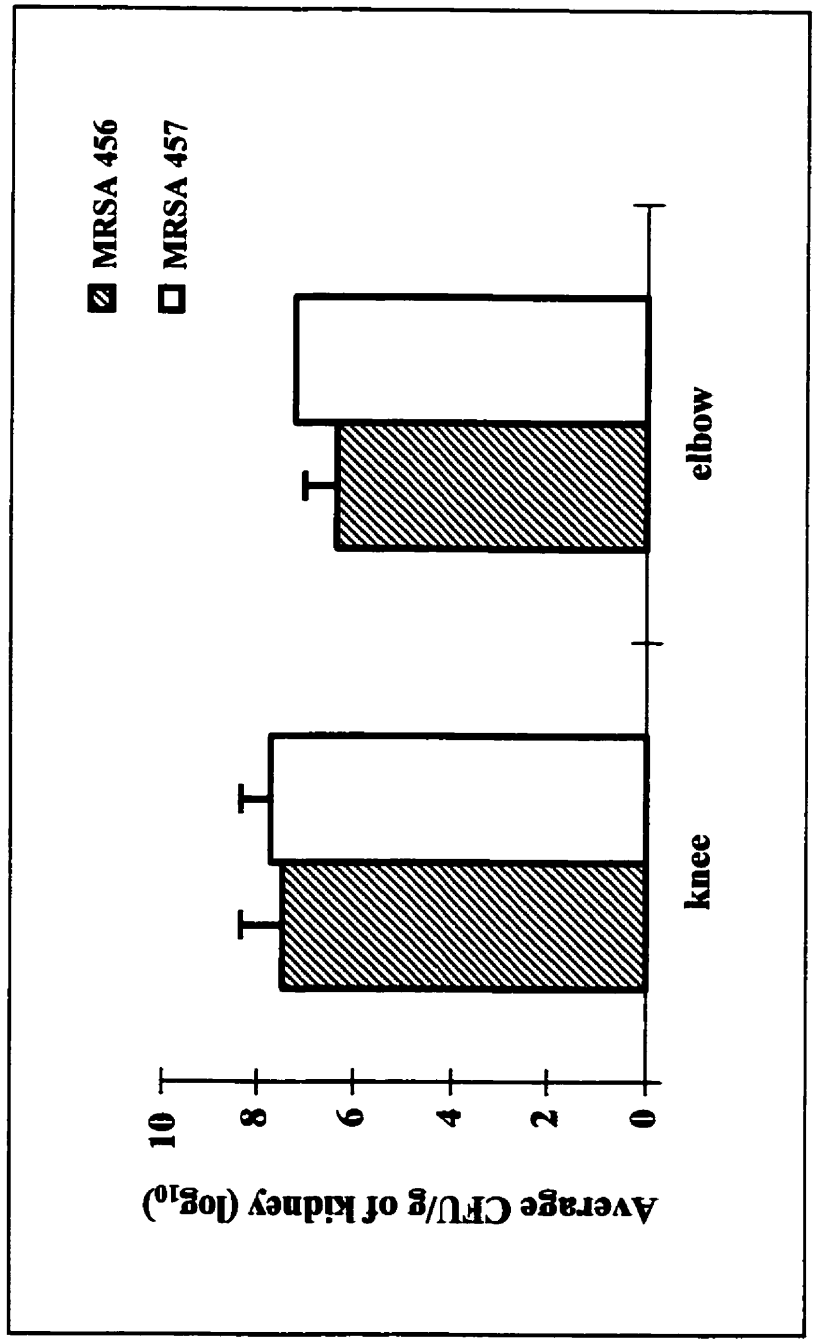


Figure 29. Joint colonization of female uPA-/- mice inoculated with MRSA

CHAPTER 4

DISCUSSION

4.1. CD1 MOUSE MODEL

4.1.1. Tissue Colonization

Following inoculation of CD1 mice with *S. aureus* it was found that the kidney, knee joint and elbow joint became chronically colonized with bacteria. *Staphylococcus aureus* has been known to cause glomerulonephritis (Sato et al., 1979; Spector et al., 1980) and previous animal models have been developed to study this disease (Dyke, 1923; Kapral, 1974; Gorrill, 1958; Lee et al., 1985). Virulence factors such as exopolysaccharide and coagulase have been suggested to aid in the development of kidney infection (Lee et al., 1985; De Navasquez, 1950; Li and Kapral, 1962). Bacterial arthritis caused by *S. aureus* is extremely prevalent (Mikhail and Alarcon, 1993). The synovium of a joint is susceptible to hematogenous seeding of bacteria because it is a highly vascular connective tissue without a basement membrane (Gentry, 1997). It is possible that bacteria hematogenously spread to the joints where specific virulence factors allow bacterial arthritis to prevail. Bremell and colleagues have shown similar results to the present findings, where in a mouse model *S. aureus* colonizes the kidney and joints (Bremell et al., 1991). Tissue colonization exhibited by the CD1 mice inoculated with *S. aureus* correlates with previous studies, which also found the mouse kidneys and joints to become colonized following bacterial inoculation. Factors

mentioned above, such as virulence factors and joint structure allow kidney and joint colonization to prevail.

Working with an animal model to perform research inevitably gives variations in results even though they may be genetically identical. Each mouse is unique and may react in a unique manner to infectious agents such as *Staphylococcus aureus*. Certain individual mice may be colonized with bacteria more than others and some mice may die whereas other mice treated in exactly the same manner may not die. The basis for such variation between animals is unknown for the most part. However, with sufficiently large experimental groups, these individual variations should not skew the final conclusions.

4.1.2. *In vitro* Urokinase Responsiveness and *In Vivo* Pathogenesis

This study demonstrates with two urokinase responsive and two urokinase non-responsive isolates, that there is little correlation between the *in vivo* pathogenesis and the *in vitro* phenomenon of bacterial UK responsiveness. MRSA 456 and MSSA HR78, the UK responsive isolates, did not display greater kidney colonization or increased mortality compared to the UK non-responsive isolates, MRSA 457 and MSSA CSA-1. The *in vitro* influence of the *S. aureus* phenotype could have been obscured *in vivo* because there may not be an excess of urokinase in the mouse model, or possibly because of overriding host factors present *in vivo* that are absent in the *in vitro* growth medium conditions. Whatever the mechanism(s), the *in vivo* data obtained suggests that the *in vitro* UK responsiveness phenotype does not overtly influence the growth and colonization of the isolates when assessed *in vivo*.

4.1.3. Pathogenesis of MRSA versus MSSA

In these studies, it was however observed that the MRSA isolates tested are less pathogenic than the MSSA isolates assessed. Significantly more deaths occurred following inoculation of CD1 mice with MSSA isolates than with MRSA isolates. These findings are consistent with studies reported by Mizobuchi and colleagues, who found that MRSA strains are less virulent than MSSA in normal hosts (Mizobuchi et al., 1994), as well as other studies indicating that the prevalence of MRSA as nosocomial pathogens was not the result of increased virulence relative to MSSA strains (Hershow et al., 1992). Hershow and colleagues suggest that MRSA prevalence may be a result of previous antibiotic use in patients and prolonged pre-infection hospital stay. Still other studies indicate that MRSA and other *S. aureus* strains have equivalent virulence, as shown by laboratory, animal model, clinical and case-control studies (Peacock et al., 1980, French et al., 1990, Peacock et al., 1981, Knayr et al., 1990). This topic remains controversial however, with some studies suggesting that MRSA produce more virulence factors such as coagulase and new types of cytotoxins (Jordens et al., 1989, Kaku and Yoshida, 1994). Muder and colleagues reported that colonization of long-term care patients with MRSA poses a significantly greater risk for infection than does colonization by MSSA (Muder et al, 1991), and it has been suggested that intensive care unit patients with MRSA have a risk of mortality three times higher than those with MSSA (Ibelings and Bruining, 1998). Thus, conclusions regarding the comparison of MRSA and MSSA virulence are still under debate and further study is needed to fully address the virulence question.

However, the present model could be used to address some of these issues and expand the current studies using additional isolates of *S. aureus*.

4.1.4. Gender Influence

An additional important concept raised by the present study was that female CD1 mice were more susceptible to MRSA colonization and mortality than their male mice counterparts. Furthermore, castration of male mice prior to sexual maturity resulted in an increase in bacterial colonization resembling a phenotype between those of intact male and female inoculated mice. This phenomenon was seen in the colonization of the kidney, knee joint and elbow joint. These results suggest that steroid hormones such as androgens and/or estrogens have an influence on the pathogenesis of the invading organism or somehow affect the host immune system. Oestrogen is known to accelerate inflammation and immune responses in both mice and humans (Carlsten et al., 1991). It has been postulated that oestrogen has a differential effect on T and B cell-mediated immune responses. Interestingly, Yirrell and colleagues found similar results to the present study when they inoculated mice with herpes simplex virus (Yirrell et al., 1987). Following intravenous inoculation with avirulent strains of herpes simplex virus, male mice were less susceptible to paralysis than female mice. Castration of male mice increased the frequency of paralysis to levels seen in the female mice and treatment with testosterone reversed this change, suggesting that testosterone is somehow acting as a protectant for the host. Testosterone has previously been shown to contribute to the prevention of infections with *S. aureus* in castrated rats (Atef and Sokkar, 1980). In a rat

model, testosterone apparently acts to protect castrated rats by acting synergistically with antibiotics to clear infection with *S. aureus*. Whether this was due to a direct effect of the hormone on the organisms or an indirect effect via host defense systems is not clear. In contrast, some studies have indicated that mortality is associated with serum testosterone levels and protection from *S. aureus* toxic shock syndrome is provided by estrogen (Best et al., 1986). Furthermore, it has been shown that treatment of male and female mice with oestrogen significantly enhanced the growth of "pyelonephritic" strains of *E. coli* in the kidney (Harle et al., 1975). Kinsman and Collard also showed that estrogen dosing following ovariectomy predisposed rats toward infection with *Candida albicans* (Kinsman and Collard, 1986). On the other hand, when progesterone was added to *Listeria monocytogenes* growth media the numbers of bacteria decreased (Basher et al., 1985). An increase in progesterone concentration in culture medium resulted in a decrease in mortality of chick embryos inoculated with *L. monocytogenes*. In the studies discussed here involving CD1 mice and *S. aureus*, testosterone could somehow be acting as a protectant to the host or acting in a harmful manner to *S. aureus* preventing bacterial growth. Estrogen could also be involved, influencing the host in a negative manner making the mice more susceptible to *S. aureus* infection. Correlating with studies by Harle and colleagues, estrogen could also be influencing the bacteria in a positive manner promoting bacterial growth and pathogenecity (Harle et al., 1975). Further studies involving sex hormones would have to be performed to determine the exact reason(s) a gender influence is seen following inoculation of mice with *S. aureus*.

4.2. UROKINASE DEFICIENT MOUSE MODEL

4.2.1. Tissue Colonization

Bacterial colonization shown in the UK deficient mouse model was similar to what was displayed in the CD1 mice. Colonization of the heart or endocarditis was however more common in the knockout mice. This is not surprising as *S. aureus* accounts for 25 to 35 percent of endocarditis cases (Sanabria et al., 1990; Sandre and Shafran, 1996). Endocarditis often appears in people with underlying immune complications which could be why heart colonization was seen more frequently in uPA-/- mice than in CD1 mice. Urokinase deficient mice have been shown to be more susceptible to certain infectious conditions than normal background matched mice (Gyetko et al., 1996; Shapiro et al., 1997; Beck et al., 1999). However, as the genetic backgrounds of the CD1 and uPA-/- mice are different, further investigations with uPA-/- and uPA+/+ of the same genetic background will be required to make stronger conclusions in this area.

4.2.2. Inoculum Dosage

The optimal inoculation dose of bacteria to develop kidney colonization with a low mortality rate was 10^7 CFU per mouse. Bacterial inoculation involving doses less than 10^7 CFU resulted in cases of low bacterial colonization to no colonization. Mice inoculated with 10^4 CFU or lower usually had no detectable bacterial colonization. One of the main problems in developing animal models with *S. aureus* is the resistance of test animals to challenge with this microorganism (Adlam et al., 1983). Inoculations of mice

with over 10^7 CFU per mouse resulted in death. Gorrill found with the same inoculation route as used in the present studies, the ID_{50} to be approximately $5x10^6$ CFU (Gorrill, 1958). Many of the previous animal models developed to study *S. aureus* infections have used an inoculation dose of approximately 10^7 CFU per mouse (Lee et al., 1985; Abdelnour et al., 1994; Nilsson et al., 1999) or higher (Bremell et al., 1992; Bremell et al., 1991). This correlates with the findings in the present studies, where 10^7 CFU per mouse, in both male and female mice, was found to be the optimal inoculation dosage.

4.2.3. *In Vitro* Urokinase Responsiveness and *In Vivo* Pathogenesis

Inoculation of the uPA-/- mice with bacteria differing in *in vitro* UK responsiveness did not seem to overtly effect the *in vivo* pathogenesis of the isolate. This is true when looking at kidney and joint colonization with both MRSA and MSSA isolates. These results correlate with the CD1 mouse model results discussed earlier. When specifically focusing on kidney colonization it was shown that MRSA 457, which is the *in vitro* UK non-responsive isolate, had consistently higher colonization levels than MRSA 456, but the differences were not significant. However, when looking at the colonization of male survivors of MRSA inoculation, MRSA 456 had significantly higher levels of colonization than MRSA 457. The survival rate of mice inoculated with MRSA 456 or MRSA 457 was however comparable. If host UK availability was the only factor considered, it would be expected that both MRSA isolates would behave in the same manner, because there is no uPA present in these mice. This would eliminate the opportunity for the observation of differing UK responsiveness phenotypes in the *S.*

aureus isolates. Using the *in vivo* model requires the consideration of many host factors which interact together in a complex manner. If uPA+/+ background matched mice were used as controls, the direct influence of host UK on bacterial pathogenesis could be determined in more detail, as mentioned earlier.

4.2.4. Pathogenesis of MRSA versus MSSA

With the uPA-/- mice, it was again observed that the MRSA isolates tested are less pathogenic than the MSSA isolates assessed. More deaths occurred following inoculation of uPA-/- mice with MSSA isolates than with MRSA isolates. These findings are consistent with other studies which found MRSA strains to be less virulent than MSSA strains in normal hosts (Mizobuchi et al., 1994), and indicate that the prevalence of MRSA as nosocomial pathogens was not the result of increased virulence relative to MSSA strains (Hershow et al., 1992). As mentioned previously, the debate regarding the relative virulence of MRSA and MSSA is still ongoing.

It was shown that uPA-/- mice are more susceptible to death than CD1 mice following inoculation of *S. aureus*. Because background matched uPA+/+ mice were not used as controls, any conclusions drawn from this observation are purely speculations. The loss of uPA in mice has however been shown to make the mice more susceptible to certain infectious conditions (Gyetko et al., 1996; Shapiro et al., 1997; Beck et al., 1999; Busso et al., 1998). Urokinase can help to activate the complement system and trigger the accumulation of PMNs ultimately helping to elicit an immune response (Christman et al., 1977). Urokinase is also expressed by macrophages and active lymphocytes. The

receptor for UK, urokinase plasminogen activator receptor (uPAR), clusters to the leading edge of migrating monocytes during chemotaxis therefore suggesting the use of UK in monocyte migration. Because of an impaired immune system, *S. aureus* isolates may be able to flourish in uPA-/- mice. Host defense responses in uPA-/- mice may not be as vigorous as in normal mice making the uPA-/- mice more susceptible to infections. This would explain the increase in susceptibility to *S. aureus* infection displayed by uPA-/- mice compared to CD1 mice. The lack of complete activation of essential host immune components such as PMNs and macrophages would allow bacterial survival and subsequent disease conditions to prevail.

4.2.5. Gender Influence

Female uPA-/- mice were shown to have significantly higher levels of colonization than male mice in the survival colonization studies performed with MRSA 457. Inoculation of mice with MSSA isolates did not display a gender difference but this may be because of the extremely low survival rate of mice inoculated with MSSA isolates. The immune systems of males and females have been shown to be inherently different (Staples et al., 1999). Experimentally, sex hormones have been shown to directly effect the development of different diseases (Atef and Sokkar, 1980; Best et al., 1986; Yirrell et al., 1987; Carlsten et al., 1991). As discussed in detail earlier, sex hormones can act in a harmful or beneficial manner depending on the specific situation. Female uPA-/- mice could generally be more susceptible to *S. aureus* infections than background matched male mice. The results displayed here could be a result of hormonal

effect. Testosterone could be protecting male mice from *S. aureus* infections via host mechanisms or by negatively influencing bacterial growth. Estrogen may also be affecting the host in a negative manner allowing disease to occur or estrogen could somehow be increasing bacterial growth and virulence. The results achieved in these studies could also be a combination of the above mentioned situations, involving both testosterone and estrogen.

4.3. HISTOLOGICAL FINDINGS

It was found through histological analysis of kidney and joint tissues from both CD1 and uPA-/- mice, that an immune response is being mounted by the host to fight off infection. In the majority of samples, PMNs are seen in the infected tissues. Infected uPA-/- mice appeared to have bacteria in the glomeruli which was not observed with the CD1 mice. Infected glomeruli displayed no or fewer PMNs when compared to other areas of infected tissues. The lack of urokinase in the knockout mice compared to the CD1 mice may have allowed the bacteria to colonize this highly vascular area of the kidney. Bacterial microcolonies are also seen in the histological preparations, suggesting the bacteria are working in concert to evade host immune responses. This type of formation has been shown in many other infections in the form of a biofilm, and is believed to assist in the survival of the bacteria (Habash and Reid, 1999; Reid, 1999). There is also evidence of bone and cartilage destruction which could be a result of bacterial toxins released into the tissue. Bremell and colleagues also found cartilage and bone destruction following inoculation of mice with *S. aureus* (Bremell et al., 1991).

The findings mentioned above are consistent in all of the mouse experimental groups for all of the *S. aureus* tested, with minor variances between individual mice. This suggests that histologically there is no overt difference between male and female mice, *in vitro* UK responsive and UK non-responsive mice, or between MSSA and MRSA isolates.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1. SUMMARY AND CONCLUSIONS

Two animal models were developed to study the pathogenesis of *S. aureus* infections. CD1 and uPA-/- mice were inoculated with different isolates of MRSA and MSSA and analyzed for bacterial affects. It was found that the bacteria mainly colonized the kidney and knee and elbow joints. In the uPA-/- mice the heart was also shown to be colonized, but not as predominantly as the other tissues mentioned. The development of these models allowed further studies involving *S. aureus*.

The two animal models were utilized to assess the relationship between *in vitro* bacterial UK phenotype and *in vivo* pathogenesis with both MRSA and MSSA isolates. It was found that the *in vitro* UK phenotype of the isolates tested did not overtly influence *in vivo* bacterial behavior. However, it was observed that bacterial colonization and mortality by the MSSA isolates assessed was more significant than that following infection with the MRSA isolates. A gender difference in MRSA susceptibility was also

clearly evident. In the CD1 mice this phenomenon was displayed in the kidney, knee joint and elbow joint whereas in the uPA-/- mice gender influence in bacterial colonization was shown with MRSA 457 colonization of the kidney.

5.2. SUGGESTIONS FOR FURTHER WORK

This study has provided some insight into the pathogenesis of *S. aureus*, but like many scientific investigations, has also created more questions to be answered.

Further exploring the concept of gender influences in the pathogenesis of *S. aureus* could be executed through studies involving hormonal supplements or replacement. For example, castrated male mice could be given testosterone supplements and compared to non-treated castrated male and female mice. Castrating females, through ovariectomies, could be performed to determine if estrogen has an effect on bacterial pathogenesis. Normal mice could be treated with either estrogen of progesterone to conclude if excess amounts of these hormones have an influence on the development of disease. *In vitro* experiments could also be performed to determine if the addition of sex hormones to the growth media cause an increase in bacterial growth. It would also be interesting to determine if other *S. aureus* isolates and other bacterial species exhibit the same gender dependent differences in infection characteristics.

Another valuable set of experiments would involve characterizing the *S. aureus* isolates used in these experiments. Because little is known about these isolates, including the virulence factors they produce, this information would prove to be valuable in helping to explain some of the results attained. Once the bacterial virulence factors are

known, further studies could be performed to determine how specific factors influence the colonization and subsequent sequlae of the kidney and joint displayed in the model used in these studies.

Urokinase responsiveness in other bacterial species *in vitro* and *in vivo* would be another interesting route to explore. A total of four bacterial isolates were used in these studies. Analyzing other bacteria would further determine if *in vitro* UK responsiveness has an influence on *in vivo* pathogenecity and if so how.

In order to analyze the influence of host UK levels *in vivo* on bacterial pathogenesis, background matched controls could be obtained and used as controls to uPA-/- mice. These studies could be performed using *S. aureus* isolates, as well as other pathogenic bacteria.

BIBLIOGRAPHY

Abdelnour, A., Arvidson, S., Bremell, T., Ryden, C., and A. Tarkowski. 1993. The accessory gene regulator (agr) controls *Staphylococcus aureus* virulence in a murine arthritis model. Infect. Immun. **61:** 3879-3885.

Abdelnour, A., Bremell, T., Holmdahl, R., and A. Tarkowski. 1994. Role of T lymphocytes in experimental *Staphylococcus aureus* arthritis. Scand. J. Immunol. **39:** 403-408.

Adlam, C., Anderson, J.C., Arbuthnott, J.P., Easmon, C.S.F., and Noble, W.C. 1983. Animal and human models of staphylococcal infection. *In* Staphylococci and staphylococcal infections. 1. *Edited by* Academic Press Inc., London. pp. 357-384.

Akram, J., and A.E. Glatt. 1998. True Community-Acquired Methicillin-Resistant *Staphylococcus* Bacteremia. Infec. Control Hosp Epidem. **19**:106-107.

Albus, A., Arbeit, R.D., and J.C. Lee. 1991. Virulence of *Staphylococcus aureus* mutants altered in type 5 capsule production. Infect. Immun. **59:** 1008-10014.

Archer, G.L., and D.M. Niemeyer. 1994. Origin and evolution of DNA associated with resistance to methicillin in staphylococci. Trends Microbiol. **2**:343-347.

Archer, G.L., Niemeyer, D.M., Thanassi, J.A., and M.J. Pucci. 1994. Dissemination among staphylococci of DNA sequences associated with methicillin resistance. Antimicrob Agents Chemother. **38**:447-454.

Atef, M., and S.M. Sokkar. 1980. Testosterone-antibiotic effectiveness on *Staphylococcus aureus* infected rats. Clin. Exp. Pharm. Physiol. **7:** 603-607.

Atkinson, B.A., and V. Lorian. 1984. Antimicrobial agent susceptibility patterns of bacteria in hospitals from 1971 to 1982. J. Clin. Microbiol. **20:** 791-796.

Basher, H.A., Fowler, D.R., Seaman, A., and M. Woodbine. 1985. The effect of progesterone on the growth and virulence of *Listeria monocytogenes*. Zentralblatt Fur Bakteriologie, Mikrobiologie, Und Hygiene - Series A, Medical Microbiology, Infectious Diseases, Virology, Parasitology. **259:** 351-358.

Beck, J.M., Preston, A.M., and M.R. Gyetko. 1999. Urokinse-type plasminogen activator in inflammatory cell recruitment and host defense against *Pneumocystis carinii* in mice. Infec. Immun. **67:** 879-884.

Beck, W.D., Berger-Bachi, B., and F.H. Kayser. 1986. Additional DNA in methicillin-resistant *Staphylococcus aureus* and molecular cloning of mec-specific DNA. J Bacteriol. **165**:373-378.

Bennett, H.S., Wyrick, A.D., Lee, S.H., and J.H. McNeil. 1976. Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. Stain Technol. **51:** 71-97.

Best, G.K., Abney, T.O., Kling, J.M., Kirkland, J.J., and D.F. Scott. 1986. Hormonal influence on experimental infections by a toxic shock strain of *Staphylococcus aureus*. Infect. Immun. **52:** 331-333.

Boxerbaum, B., Jacobs, M., and R. Cechner. 1988. Prevalence and significance of methicillin-resistant *Staphylococcus aureus* in patients with cystic fibrosis. Ped Pulmon. **4**:159-163.

Boyle, M.D., and R. Lottenberg. 1997. Plasminogen activation by invasive human pathogens. Thrombosis and Haemostasis. **77**:1-10.

Bremell, T., Abdelnour, A., and A. Tarkowski. 1992. Histopathological and serological progression of experimental *Staphylococcus aureus* arthritis. Infec. Immun. **60:** 2976-2985.

Bremell, T., Lange, S., Holmdahl, R., Ryden, C., Hansson, G., and A. Tarkowski. 1994. Immunopathological features of rat *Staphylococcus aureus* arthritis. Infec. Immun. **62:** 2334-2344.

Bremell, T., Lange, S., Svesson, L., Jennische, Grondahl, K., Carlsten, H., and A. Tarkowski. 1990. Outbreaks of spontaneous staphylococcal arthritis and osteitis in mice. Arthritis Rheum. **33:**1739-1744.

Bremell, T., Lange, S., Yacoub, A., Ryden, and A. Tarkowski. 1991. Experimental *Staphylococcus aureus* arthritis in mice. Infec. Immun. **59:**2615-2623.

Brock, T.D. and M.T. Madigan. 1991. Biology of Microorganisms. 6th Ed. Prentice Hall, New Jersey.

Busso, N., Peclat, V., Van Ness, K., Kolodziesczyk, E., Degen, J., Bugge, T., and A. So. 1998. Exacerbation of antigen-induced arthritis in urokinase-deficient mice. J. Clin. Invest. **102:** 41-50.

Carlsten, H., Nilsson, N., Jonsson, R. and A. Tarkowski. 1991. Differential effects of oestrogen in murine lupus: acceleration of glomerulonephritis and amelioration of T cell-mediated lesions. J. Autoimmun. **4:** 845-856.

Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., De Vos, R., van den Oord, J., Collen, D., and R. Mulligan. 1994. Physiological consequences of loss of plasminogen activator gene function in mice. Nature. **368**:419-424.

Chambers, H.F. 1997. Parenteral Antibiotics for the Treatment of Bacteremia and Other Serious Staphylococcal Infections. *In* The Staphylococci in Human Disease. *Edited by* K.B. Crossley and G.L. Archer. pp. 583-601.

Christman, J., Silverstein, S., and G. Acs. 1977. Plasminogen activators. *In* Proteinases in mammalian cells and tissues. *Edited by* A.J. Barrett. Amsterdam, North-Holland. 91-149.

Christner, R.B., and M.D. Boyle. 1996. Role of staphylokinase in the aquisition of plasmin(ogen)-dependent enzymatic activity by staphylococci. J Infect Dis **173**:104-112.

Cunningham, R., Cockayne, A., and H. Humphreys. 1996. Clinical and molecular aspects of the pathogenesis of *Staphylococcus aureus* bone and joint infections. J. Med. Microbiol. **44:** 157-164.

De Navasquez, S. 1950. Experimental pyelonephritis in the rabbit produced by staphylococcal infection. J. Path. Bact. **62:** 429-437.

Dunford, C.E. 1997. Methicillin resistant *Staphylococcus aureus*. Nurs Standard. **11**:58-62.

Dyke, S.C. 1923. On the passage of the *Staphylococcus aureus* through the kidney of the rabbit. J. Path. Bact. **26:** 164-175.

Fowler, V.G., Sanders, L.L., Sexton, D.J., Kong, L., Marr, K.A., Gopal, A.K., Gottlieb, G., McClelland, R.S., and G.R. Corey. 1998. Outcome of *Staphylococcus aureus* bacteremia according to compliance with recommendations of infectious diseases specialists: experience with 244 patients. Clin. Infect. Dis. **27:** 478-486.

French, G.L., Cheng, A.F.B., Ling, J.M.L., Mo, P., and S. Donnan. 1990. Hong Kong strains of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* have similar virulence. J. Hosp. Infect. **15:** 117-125.

Gemmell, C.G., Goutcher, S.C., Reid, R., and R.D. Sturrock. 1997. Role of certain virulence factors in a murine model of *Staphylococcus aureus* arthritis. J. Med. Microbiol. **46:** 208-213.

Gentry, L.O. 1997. Osteomyelitis and Other Infections of Bones and Joints. *In* The Staphylococci in Human Disease. *Edited by* K.B. Crossley and G.L. Archer. pp. 455-474.

Goldenberg, D.L., and J.I. Reed. 1985. Bacterial arthritis. N. Engl. J. Med. **312:** 764-771.

Gorrill, R.H. 1958. The establishment of staphylococcal abscesses in the mouse kidney. Br. J. Exp. Path. **39:** 203-212.

Gyetko, M.R., Chen, G., McDonald, R., Goodman, R., Huffnagle, G.B., Wilkinson, C.C., Fuller, J.A., and G.B. Toews. 1996. Urokinase is required for the pulmonary inflammatory response to *Cryptococcus neoformans*. J Clin Invest. **97**:1818-1826.

Habash, M., and G. Reid. 1999. Microbial biofilms: their development and significance for medical device-related infections. J. Clin. Pharmacol. **39:** 887-898.

Harle, E.M.J., Bullen, J.J., and D.A. Thomson. 1975. Influence of oestrogen on experimental pyelonephritis caused by *Escherichia coli*. Lancet. **2:** 283-286.

Hart, D.A. 1992. Regulation of plasminogen activators in connective tissues: potential for thrombolytic therapy in Collagen-Vascular Diseases. Fibrinolysis. **6**(suppl.):43-48.

Hart, D.A., and A. Rehemtulla. 1988. Plasminogen activators and their inhibitors: regulators of extracellular proteolysis and cell function. Comp Biochem Physiol **90B**:691-708.

Hart, D.A., and D.E. Woods. 1994. Human urokinase, a serine protease, potentiates the in-vitro growth of micro-organisms which commonly infect burn patients. J Med Microbiol **41**:264-271.

Hart, D.A., Louie, T., Krulicki, W., and C. Reno. 1996. *Staphylococcus aureus* strains differ in their *in vitro* responsiveness to human urokinase: evidence that methicillin-resistant strains are predominantly nonresponsive to the growth-enhancing effects of urokinase. Can J Microbiol. **42**:1024-1031.

Hershow, R.C., Khayr, W.F., and N.L. Smith. 1992. A comparison of clinical virulence of nosocomially acquired methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* infections in a university hospital. Infect. Control Hosp. Epidemiol. **13:** 587-93.

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and F.C. Tenover. 1997. Methiciliin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility . J. Antimicrob. Chemother. **40:** 135-136.

Ibelings, M.M., and H.A. Bruining. 1998. Methicillin-resistant *Staphylococcus aureus*: acquisition and risk of death in patients in the intensive care unit. Eur. J. Surg. **164:** 411-418.

Ing, M.B., Baddour, L.M., and A.S. Bayer. 1997. Bacteremia and Infective Endocarditis: Pathogenesis, Diagnosis, and Complications. *In* The Staphylococci in Human Disease. *Edited by* K.B. Crossley and G.L. Archer. pp. 331-354.

Ingerman, M.J., and J. Santoro. 1989. Vancomycin: a new old agent. Infect. Dis. Clin. North Am. **3:** 641-651.

Jacoby, G.A., and G.L. Archer. 1991. New mechanisms of bacterial resistance to antimicrobial agents. New Eng J Med. **324**:601-612.

Jordens, J.Z., Duckworth, G.J., and R.J. Williams. 1989. Production of 'virulence factors' by 'epidemic' methicillin-resistant *Staphylococcus aureus* compared with those of methicillin-sensitive strains. J. Med. Microbiol. **30:** 245-252.

Kaku, M., and R. Yoshida. 1994. Virulence factors of drug resistant strain. Nipon Rinsho - Jap. J. Clin. Med. **52:** 322-326.

Kapral, F.A. 1974. *Staphylococcus aureus*; some host-parasite interactions. Ann. N. Y. Acad. Sci. **236:** 267-274.

Keystone, E.C., Schorlemmer, H.U., Pope, C., and C. Allison. 1977. Zymosan-induced arthritis. Arthritis Rheum. **20:** 1396-1401.

Kinsman, O.S. and A. E. Collard. 1986. Hormonal factors in vaginal candidiasis in rats. Infect. Immun. **53:** 498-504.

Knayr, W., Hershow, R., and N. Smith. 1990. A comparison of the virulence of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* infections. Proc. Interscience. Conf. Antimicrob. Agents and Chemother. 30th, 1990. Abstr. p.468.

Koyama, A., Kobayashi, M., Yamaguchi, N., Yamagata, K., Takano, K., Nakajima, M., Irie, F., Goto, M., Igarashi, M., Iitsuka, T, Aoki, Y., Sakurai, H., Sakurayama, N., and K. Fukao. 1995. Glomerulonephritis associated with MRSA infection: a possible role of bacterial superantigen. Kid. International. **47:** 207-216.

Labischinski, H. 1992. Consequences of the interaction of B-lactam antibiotics with penicillin binding proteins from sensitive and resistant *Staphylococcus aureus* strains. Med Microbiol Immunol. **181**:241-265.

Lee, J.C., Betley, M.J., Hopkins, C.A., Perez, N.E., and G.B. Pier. 1987. Virulence studies, in mice, of transposon-induced mutants of *Staphylococcus aureus* differing in capsule size. J. Infect. Dis. **156:** 741-50.

Lee, J.C., Hopkins, C.A., and G.B. Pier. 1985. The role of the *Staphylococcus aureus* capsule in abscess formation. Zentralbl. Bakteriol. Mikrobiol. Hyg. **14** (Suppl.): 219-224.

Lee, J.C., Perez, N.E., and C.A. Hopkins. 1989. Production of toxic shock syndrome toxin 1 in a mouse model of *Staphylococcus aureus* abscess formation. Rev. Infect. Dis. **11** (Suppl. 1): S254-S259.

Levy, S.B. 1998. The challenge of antibiotic resistance. Scientific American. March 1998, 46-53.

Li, I.W., and F.A. Kapral. 1962. Virulence and coagulases of *Staphylococcus aureus*. II. Survival of certain coagulase-negative mutants in the organs of intravenously infected rabbits. J. Infect. Dis. **111:** 204-208.

Lowy, F.D. 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **339:** 520-532.

Marantz, P.R., Linzer, M., Feiner, C.J., Feinstein, S.A., Kozin, A.M., and G.H. Friedland. 1987. Inability to predict diagnosis in febrile intravenous drug abusers. Ann. Intern. Med. **106:** 823-828.

Mikhail, I.S., and G.S. Alarcon. 1993. Nongonococcal bacterial arthritis. Rheum. Dis. Clin. North Am. **19:** 311-331.

Mizobuchi, S., Minami, J., Jin, F., Matsushita, and A. Okabe. 1994. Comparison of the virulence of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*. Micro. Immunol. **38:** 599-605.

Muder, R.R., Brennen, C., Wagener, M.M., Vickers, R.M., Rihs, J.D., Hancock, G.A., Yee, Y.C., Miller, J.M., and V.L. Yu. 1991. Methicillin-resistant staphylococcal colonization and infection in a long-term care facility. Ann. Intern. Med. **114:**107-112.

Musser, J.M., and V. Kapur. 1992. Clonal analysis of methicillin-resistant *Staphylococcus aureus* strains from intercontinental sources: association of the *mec* gene with divergent phylogenetic lineages implies dissemination by horizontal transfer and recombination. J Clin Microbiol. **30**:2058-2063.

Nilsson, I.M., Hartford, O., Foster, T., and A. Tarkowski. 1999. Alpha-toxin and gamma-toxin jointly promote *Staphylococcus aureus* virulence in murine septic arthritis. Infec. Immun. **67:** 1045-1049.

Nilsson, I.-M., Patti, J.M., Bremell, T., Hook, M., and A. Tarkowski. 1998. Vaccination with recombinant fragment of collagen adhesin provides protection against *Staphylococcus aureus*-mediated septic death. J Clin Invest. **101**:2640-2649.

Olson, M.E., and J. Bruce. 1986. Ovariectomy, ovariohysterectomy and orchidectomy in rodents and rabbits. Can. Vet. J. **27:** 523-527.

Peacock, J.E., Jr., Moorman, D.R., Wenzel, R.P., and G.L. Mandell. 1981. Methicillin-resistant *Staphylococcus aureus*: microbiological characteristics, antimicrobial susceptibilities, and assessment of virulence of an epidemic strain. J. Infect. Dis. **144:** 575-582.

Peacock, J.E., Marsik, F.J., and R.P. Wenzel. 1980. Methicillin-resistant *Staphylococcus aureus*: introduction and spread within a hospital. Ann. Intern. Med. **93:** 526-532.

Piras, G., Raze, D., el Kharroubi, A., Hastir, D., Englebert, S., Coyette, J., and J.M. Ghuysen. 1993. Cloning and sequencing of the low-affinity penicillin-binding protein 3r-encoding gene of *Enterococcus hirae* S185: modular design. J. Bacteriol. **175:** 2844-52.

Projan, S.J., and R.P. Novick. 1997. The Molecular Basis of Pathogenicity. *In* The Staphylococci in Human Disease. *Edited by* K.B. Crossley and G.L. Archer. pp. 55-81.

Pujol, M., Pena, C., Pallares, R., Ayats, J., Ariza, J., and F. Gudiol. 1994. Risk factors for nosocomial bacteremia due to methicillin-resistant *Staphylococcus aureus*. Eur. J. Clin. Microbiol. Infect. Dis. **13:** 96-102.

Reid, G. 1999. Biofilms in infectious diseases and on medical devices. Int. J. Antimicrob. Agents. **11:** 223-226.

Salyers, A.A. and D.D. Whitt. 1994. Bacterial Pathogenesis: A Molecular Approach. American Society for Microbiology, Washington, DC.

Sanabria, T.J., Alpert, J.S., Goldberg, R., Pape, L.A., and S.H. Cheeseman. 1990. Increasing frequency of staphylococcal infective endocarditis: experience at a university hospital, 1981 through 1988. Arch. Intern. Med. **150:** 1305-1309.

Sandre, R.M., and S.D. Shafran. 1996. Infective endocarditis: review of 135 cases over 9 years. Clin. Infect. Dis. **22:** 276-286.

Sato, M., Nakazora, H., and T. Ofuji. 1979. The pathogenic role of *Staphylococcus aureus* in primary human glomerulonephritis. Clin. Nephrol. **11:** 190-195.

Shapiro, R.L., Duquette, J.G., Nunes, I., Roses, D.F., Harris, M.N., Wilson, E.L., and D.B. Rifkin. 1997. Animal model: Urokinase-type plasminogen activator deficient mice are predisposed to staphylococcal botryomycosis, pleuritis, and effacement of lymphoid follicles. AJP. **150**:359-369.

Siu, A.C. 1994. Methicillin-resistant *Staphylococcus aureus*: do we just have to live with it? Brit J Nurs. 3:753-758.

Smith, I.M., Wilson, A.P., Hazard, E.C., Hummer, W.K., and M.E. Dewey. 1960. Death from staphylococci in mice. J. Infect. Dis. **107:** 369-378.

Smith, J.M., and R.J. Dubos. 1956. The behavior of virulent and avirulent staphylococci in the tissues of normal mice. J. Exp. Med. **103:** 87-118.

Smith, T.L., Pearson, M.L., Wilcox, K.R., Cruz, C., Lancaster, M.V., Robinson-Dunn, B., Tenover, F.C., Zervos, M.J., Band, J.D., White, E., and W.R. Jarvis. 1999. Emergence of vancomycin resistance in *Staphylococcus aureus*. N. Engl. J. Med. **340:** 493-501.

Spector, D.A., Millan, J., Zauber, N., and J. Burton. 1980. Glomerulonephritis and *Staphylococcus aureus* infections. Clin. Nephrol. **14:** 256-261.

Staples, J.E., Gasiewicz, T.A., Fiore, N.C., Lubahn, D.B., Korach, K.S., and A.E. Silverstone. 1999. Estrogen receptor alpha is necessary in thymic development and estradiol-induced thymic alterations. J. Immunol. **163:** 4168-4174.

Stone, S.P. 1997. Managing methicillin-resistant *Staphylococcus aureus* in hospital: the balance of risk. Age Ageing. **26:** 165-168.

Tenover, F.C., Lancater, M.V., Hill, B.C., Steward, C.D., Stocker, S.A., Hancock, G.A., O'Hara, C.M., McAllister, S.K., Clark, N.C., and K. Hiramatsu. 1998. Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. J. Clin. Microbiol. **36:** 1020-1027.

Voss, A., Milatovic, D., Wallrauch-Schwarz, C., Rosdahl, V.T., and I. Braveny. 1994. Methicillin-resistant *Staphylococcus aureus* in Europe. Eur J Clin Microbiol Dis. **13**:50-55.

Wagner, S.N., Atkinson, M.J., Wagner, C., Hofler, H., Schmitt, M., and O. Wilhelm. 1996. Sites of urokinsae-type plasminogen activator expression and distribution of its receptor in the normal human kidney. Histochem Cell Bio. **105**:53-60.

Warren, G.H., Gray, J., and S.K. Vernon. 1970. The *in vitro* action of mammalian sex hormones on *Staphylococcus aureus*. Chemo. **15:** 273-285.

Wilkinson B.J., and M.J. Nadakavukaren. 1983. Methicillin-resistant septal peptidoglycan synthesis in a methicillin-resistant *Staphylococcus aureus* strain. Antimicrob. Agents Chemother. **23:** 610-613.

Yirrell, D.L., Blyth, W.A., and T.J. Hill. 1987. The influence of androgens on paralysis in mice following intravenous inocualtion of herpes simplex virus. J. Gen. Virol. **68:** 2461-2464.

Yoh, K., Kobayashi, M., Hirayama, A., Hirayama, K., Yamaguchi, N., Nagase, S., and A. Koyama. 1997. A case of superantigen-related glomerulonephritis after methicillin-resistant *Staphylococcus aureus* (MRSA) infection. Clin. Nephrol. **48:** 311-316.

Yung, R.L. 1999. Mechanisms of Lupus: The role of estrogens. Clin. Exptl. Rheumotol. **17:** 271-275.

Zavizion, B., White, J.H., and A.J. Bramley. 1997. *Staphylococcus aureus* stimulates urokinase-type plasminogen activator expression by bovine mammary cells. J. Infect. Dis. **176:** 1637-1640.