Detection and characterization of staphylococcal pathogens in the environment: a community approach

Issmat I. Kassem

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A Dissertation

Entitled


By

Issmat I. Kassem

Submitted in partial fulfillment of the requirements for

The Doctor of Philosophy in Biology (Ecology-Track)

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May 2009
An Abstract of


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Submitted in partial fulfillment of the requirements for
The Doctor of Philosophy in Biology (Ecology-Track)

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Under conditions of compromised immunity, certain pathogenic strains of staphylococci can cause severe infections in humans and animals. Of the ca. 31 species of staphylococci currently recognized, 15 are potentially pathogenic to humans. Resistance to a variety of antibiotics has given certain members of the staphylococci a prominent place among emerging and re-emerging pathogens. Staphylococcus spp are ubiquitously distributed in a variety of hosts, multiple routes of transmission between hosts and between hosts and their environment have contributed to the spread of staphylococci beyond the boundaries of health care facilities and into the community.

Environmental and community reservoirs (e.g. shared surfaces and natural environment) of staphylococci, including S. aureus and coagulase-negative staphylococci CNS, have not been thoroughly identified. Furthermore, studies related to the contamination of surfaces (clinical or environmental) with staphylococci have historically
focused on one or several species that were considered clinically important (e.g. methicillin-resistant \textit{S. aureus}, MRSA).

To address this void in our knowledge of an important group of potentially pathogenic bacteria, research was designed to:

i- identify public surfaces that harbor antibiotic-resistant bacteria communities, including several members of the staphylococci,

ii- show that \textit{mecA} (an important genetic determinate that confers resistance to several popular, therapeutic antibiotics) is widely distributed in nonstaphylococcal pathogens inhabiting a secondary environment, and;

iii- demonstrate a newly developed methodology that allows the simultaneous detection and species identification of most of the clinically important staphylococci, and apply the method to investigate the contamination of clinical surfaces with staphylococci.

In the first study, multiplex-PCR techniques were developed to detect and identify staphylococci, including methicillin-resistant staphylococci, on computer keyboard surfaces used by students and staff in a metropolitan university. The results of this study showed that computer keyboards were reservoirs for clinically important staphylococci, including methicillin-resistant \textit{S. aureus} (MRSA), methicillin-resistant \textit{S. epidermidis} (MRSE) and \textit{S. hominis} (MRSH). Consequently, this study identified public computer keyboards and similar tactile surfaces that are ubiquitous not only in the community but also in the healthcare facilities as an important reservoir for staphylococci and potential source of infection. Additionally, evidence was provided to warrant further studies to identify contaminated surfaces in the community.
In the second study, fresh recreational waters that were impacted by fecal pollution and consequently important in spreading infections in swimmers were investigated for the presence of staphylococci. The role of fresh water as a potential reservoir was not previously investigated. While antibiotic-resistant staphylococci were not isolated from the fresh water tested in this study, other antibiotic-resistant bacteria were isolated, including *P. vulgaris*, *M. morganii*, and *E. faecalis* all of which are known human pathogens. These pathogens were multi-antibiotic resistant and carried *mecA*, a gene conferring resistance to a wide range of ß-lactam antibiotics, which was previously associated only with staphylococci. The results of this study showed that *mecA* is widespread in the environment than previously thought (previous studies associated a *mecA* homologue to naturally occurring non-*aureus* staphylococci). The isolation of bacteria that were resistant to multiple clinically important antibiotics, including vancomycin and methicillin, and carried the *mecA* from a secondary environment was of particular significance, as these species (especially *E. faecalis*) are more commonly associated with a clinical environment.

In the third study, a community fingerprinting method (DGGE) was customized to allow the specific and sensitive detection and characterization of assemblages of *Staphylococcus* species that contaminated surfaces in the clinical environment. DGGE analysis showed that most surfaces in an isolation room housing a patient were contaminated with single or multiple species of clinically important staphylococci, including *S. aureus*, *S. epidermidis*, *S. simulans*, *S. hominis*, and *S. lugdunensis*. DGGE analysis was not only successful in detecting the contaminating species, but also highlighted the prevalence and identity of these species in the clinical environment.
Furthermore, DGGE analysis showed that daily room cleaning procedures were not sufficient to eradicate all staphylococci. This is an important finding, as now healthcare staff have a tool with which they can assess prevalence of contamination in their facilities and the efficiency of contamination management, allowing for a healthier patient environment.

Finally, this thesis represented studies concerned with i- aspects of public health microbiology, ii- attempts to develop methodologies to identify pathogen reservoirs in environmental and clinical matrices, and iii- achieving a better understanding of contamination with staphylococci. The methods and findings reported in this thesis can be used to further expand future studies of contamination with staphylococci in both environmental and clinical settings.
Dedication

To whom I will always return humbled….

To the universe that is warmth to our bleak world…

To whom I owe the humanity that still burn in my soul…

To the love that holds truth…..

To you: Al-Mortada (A.S.)
Acknowledgements

I am to acknowledge here those who helped me finish this journey. They are many and my words, any words when uttered aloud or written, are feeble. I owe all the bliss that comes with experiencing this world to God. His grace has sustained me and carried me all my life.

My research committee: I thank you all for being always there to help and advise me. I thank my PhD advisor, Dr. Von Sigler, for his support and encouragement throughout the past years. Perhaps, the thing I like the most is his enthusiasm and willingness to pursue new ideas. I thank Dr. Scott Heckathorn, Dr. Cyndee Gruden, Dr. Mike Weintraub, and Dr. Ron Turco for all there help, sound advice and willingness to offer their time. It would be amiss if I forget to specifically thank Dr. Heckathorn and Dr. Gruden for the opportunity to collaborate with them, it was an enjoyable experience.

My family: I thank my brothers Imad Kassem and Ali Kassem for always being there for me (albeit they have their own “unusual” way of showing it, Yes! I am still at school, and No! I will not make a lot of money). Perhaps, the hardest part about my PhD was being away from them and their families (my nephews and niece). My life-long friends: Hadi M. Yassine and Ali Al-Toufaily, thank you for your friendship and the memories. It has been fourteen years and who among us would have guessed the future of those young men.

My parents: I can not possibly thank you enough. For long, you were both my joy and sorrow. This, for what it is worth, is for you both. I am blessed to have you in my life
and dream the impossible, that someday I will be able to give back some of what you have generously and earnestly given.

My wife: Malak, I thank you for always being there and for carrying many burdens. This thesis and many other things would, truly, have not been without your help. I owe you a lot, all that I can give is my heart.
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Chapter One

Introduction

Methicillin Resistant Staphylococci: A Preview

The staphylococci are gram-positive bacteria that often occur as a part of the harmless bacterial community inhabiting the skin and nasal cavities of humans and animals (Kloos and Bannerman, 1994; Huebner and Goldmann, 1999; Hanssen and Ericson Sollid, 2006; Martins and Cunha, 2007). However, under conditions of compromised immunity, certain pathogenic strains of staphylococci can cause severe infections in humans and animals (Table 1.1) (Kloos and Bannerman, 1994; Huebner and Goldmann, 1999; Martins and Cunha, 2007 and references therein). Of the ca. 31 species of staphylococci currently recognized, 15 are potentially pathogenic to humans (Table 1.2) (Kloos and Bannerman, 1994; Martins and Cunha, 2007). The staphylococci have gained notoriety as an important cause of hospital-associated infections that are recalcitrant to typical antibiotic treatments. For example, the acquisition of the meca gene, which codes for the penicillin-binding protein 2a (PBP 2a), by staphylococci can lead to broad resistance to the ß-lactam antibiotics, including penicillins, cephalosporins and carbapenems (Hanssen and Ericson Sollid, 2006; Chambers, 2001; Delencastre et al., 2007). Resistance to a variety of antibiotics has given certain members of the staphylococci (including S. aureus and S. epidermidis) a prominent place among emerging and re-emerging pathogens (Martins and Cunha, 2007).
Table 1.1: Selected *Staphylococcus* species and the infections that they cause in humans and animals (Adapted from Lowy, 1998; Leonard and Markey, 2008; Costa et al., 1999; Katneni and Hedayati, 2007; Contreras et al., 1999; Cunha et al., 2007; Chaves et al., 2005; Iyer et al., 2005; Loeffler et al., 2007; Simango, 2005; Falcone et al., 2007; Barigye et al., 2007; Cimiotti et al., 2007; Espino et al., 2006; Chen et al., 2007). Animal-associated infections are included within brackets.

<table>
<thead>
<tr>
<th><em>Staphylococcus</em> spp</th>
<th>Examples of Bacterial Infections</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>Skin and soft tissue infections, bacteremia, pneumonia, meningitis and other diseases (mastitis and wound infections)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>Bacteremia and endocarditis (intramammary infections in goats and mastitis in bovines)</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>Endocarditis, sepsis in neonates and endophthalmitis</td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td>Central nervous system infections and primary ventriculitis (animal skin and ear infections <em>e.g.</em> pyoderma, pododermatitis and chronic otitis in dogs and cats)</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>Urinary tract infections and endocarditis</td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>Infections in neonates (bovine abortion; meningoencephalitis in a dog)</td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>Endocarditis, peritonitis, septic shock, urinary tract infection and pelvic inflammation (fatal exudative epidermitis in piglets)</td>
</tr>
</tbody>
</table>

Since *Staphylococcus* spp are ubiquitously distributed in a variety of hosts, multiple routes of transmission between hosts (zoonoses/anthroponosis) and between hosts and their environment have contributed to the spread of staphylococci (including antibiotic resistant and susceptible strains) beyond the boundaries of health care facilities and into the community. During the past decades, certain staphylococci, particularly methicillin-resistant *S. aureus* (MRSA), have become a significant health burden, claiming lives of patients, causing disabilities and costing health institutes millions of dollars in extended patient treatment and decontamination efforts.
Table 1.2: A list of several *Staphylococcus* species and their hosts. Transient hosts are enclosed with brackets. Adapted from Kloos and Bannerman (1994).

<table>
<thead>
<tr>
<th><em>Staphylococcus</em> spp</th>
<th>Natural Hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>Humans, mammals, birds</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>Humans (domestic mammals)</td>
</tr>
<tr>
<td><em>S. capitis</em></td>
<td>Humans, some primates</td>
</tr>
<tr>
<td><em>S. caprae</em></td>
<td>Humans, goats</td>
</tr>
<tr>
<td><em>S. saccharolyticus</em></td>
<td>Humans</td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>Humans, primates, domestic mammals</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>Humans, primates, (domestic mammals)</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>Humans</td>
</tr>
<tr>
<td><em>S. lugdunensis</em></td>
<td>Humans</td>
</tr>
<tr>
<td><em>S. auricularis</em></td>
<td>Humans</td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>Humans, primates</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>Humans, mammals</td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>Humans, mammals, birds</td>
</tr>
<tr>
<td><em>S. arlettae</em></td>
<td>Mammals, birds</td>
</tr>
<tr>
<td><em>S. equorum</em></td>
<td>Horses, cattle</td>
</tr>
<tr>
<td><em>S. kloosii</em></td>
<td>Mammals</td>
</tr>
<tr>
<td><em>S. gallinarum</em></td>
<td>Poultry, birds</td>
</tr>
<tr>
<td><em>S. muscae</em></td>
<td>Domestic mammals, (flies)</td>
</tr>
<tr>
<td><em>S. felis</em></td>
<td>Cats</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>Humans, mammals</td>
</tr>
<tr>
<td><em>S. carnosus</em></td>
<td>Meat and fish products, unknown</td>
</tr>
<tr>
<td><em>S. piscifermentans</em></td>
<td>Fermented fish</td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td>Mammals, birds</td>
</tr>
<tr>
<td><em>S. delphini</em></td>
<td>Dolphins</td>
</tr>
<tr>
<td><em>S. schleiferi</em></td>
<td>Human infections, unknown, dogs</td>
</tr>
<tr>
<td><em>S. hyicus</em></td>
<td>Pigs, cattle, goats</td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>Cattle, horses, goats</td>
</tr>
<tr>
<td><em>S. caseolyticus</em></td>
<td>Cattle, whales</td>
</tr>
<tr>
<td><em>S. lentus</em></td>
<td>Domestic mammals, dolphins</td>
</tr>
<tr>
<td><em>S. vitiatus</em></td>
<td>Meat products, domestic mammals, whales</td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>Mammals, birds</td>
</tr>
</tbody>
</table>

Efforts to determine the parameters that influence the emergence of staphylococcal pathogens, their virulent properties, and the affected human population have been increasing in order to eradicate these bacteria from nosocomial and community settings. However, epidemiological and decontamination studies concerning staphylococci are complicated by several factors, which include the following:

i- staphylococci species of lesser clinical value can serve as a repository of virulence genes (*e.g.* antibiotic-resistance) that can be transferred to clinically
important species through horizontal gene transfer (Hanssen and Ericson Sollid, 2006);

ii- staphylococci are ubiquitous in and on the human body and can be readily acquired or transmitted to the environment and other hosts (Guyot and Layer, 2006; Boyce, 2007), and;

iii- due to limitations in available methodologies and the erroneous assumption that *S. aureus* is the only pathogenic *Staphylococcus* species, investigations have historically focused on a single or few species of interest (Huebner and Goldmann, 1999).

While the detection and identification of each clinically important species of staphylococci individually would enhance efforts to limit the morbidity and mortality associated with the staphylococci, methodologically this is impractical. Contemporary biochemical (Kloos and Wolfhohl, 1982) and molecular identification techniques (*e.g.* polymerase chain reaction-based methods) are limited to the identification of either one (*e.g.* Brakstad *et al.*, 1993; Martineau *et al.*, 1996 and 1998) or few species (Morot-Bizot *et al.*, 2004). Furthermore, without resorting to complex and expensive approaches (*e.g.* nucleotide probes and gene sequencing (Martineau *et al.*, 2001; Heikens *et al.*, 2005), current methods can fail to provide clear differentiation between all staphylococcal species (Kloos and Bannerman, 1994 and 1995). Therefore, developing a new approach that allows the simultaneous detection and species identification of all or most of the
clinically important staphylococci occurring in a sample might be an improvement over classical approaches.

**Health risks associated with *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA)**

*Staphylococcus aureus* is the most studied *Staphylococcus* spp in clinical and community settings and the most problematic of the staphylococci in terms of public health importance. For example, *S. aureus* is regarded as a primary cause of healthcare-associated infections (Lowy, 1998; Zetola *et al.*, 2005), affecting close to 2 million patients in the US yearly (CDC, 2002). Approximately 20% of humans are permanent carriers of *S. aureus*, while 60% of the population carries the bacterium intermittently (Foster, 2004). *S. aureus* predominantly colonizes the nares and can also occur in/on other body locations, including the groin, vagina, pharynx, and the armpits. Carriers of *S. aureus* are at increased risk of infection (Lowy, 1998). For example, under conditions of compromised immunity (e.g. HIV, diabetes, hemodialysis), opportunistic *S. aureus* can cause skin and soft tissue infections in addition to more serious respiratory (pneumonia), circulatory (bacteremia), central nervous system (meningitis) and other diseases (Lowy, 1998; Klevens *et al.*, 2007).

The success of *S. aureus* as a pathogen can be partially attributed to an arsenal of proteins that not only facilitate the colonization of damaged tissues and help the bacterium to avoid host immune-response antibodies, but also act as toxins that damage host cell membranes (Foster, 2004). Of primary public health concern is methicillin resistant *S. aureus* (MRSA), which exhibits resistance to an array of antibiotics, including
penicillin, methicillin, oxacillin and others (Zetola et al., 2005), and is responsible for an increased number of infections that resist traditional chemotherapy (Fig. 1.1). Today, MRSA is responsible for approximately 43% of all *S. aureus*-related hospitalizations in the US (Noskin et al., 2007), and was associated with 18,650 death cases in 2005 (Klevens et al., 2007). In the UK, deaths resulting from MRSA have risen from 51 in 1993 to 800 in 2003 (www.medicalnewstoday.com), while recorded cases of MRSA infections increased 2300% during the same period.

![Graph of S. aureus infections](image)

**Figure 1.1:** From Lowy, 1998; *S. aureus* infections in Intensive Care Units as reported in the National Nosocomial Infections Surveillance System. ○: infections caused by *S. aureus*, ■: infections with methicillin-resistant *S. aureus*, and ●: infections with methicillin-resistant *S. aureus* that were sensitive to vancomycin but resistant to other antibiotics, including gentamicin, tobramycin, amikacin, ciprofloxacin, clindamycin, erythromycin, chloramphenicol, and trimethoprim–sulfamethoxazole.
In response to the dramatic increases in penicillinase-producing *S. aureus* isolates that exhibited high resistance to penicillin, a new antibiotic, methicillin (a semi-synthetic penicillinase-resistant penicillin) was introduced in 1959 (Chambers, 1988). Although resistance to methicillin was initially observed in staphylococci other than *S. aureus*, the first strains of methicillin resistant *S. aureus* were reported in England within several months of methicillin’s introduction (Jevons, 1961). However, the clinical significance of these isolates was regarded with uncertainty (Chambers, 1988), and it was not until the late 1960s that MRSA outbreaks were initially reported in scientific literature (Barrett *et al.*, 1968; Panlilio *et al.*, 1992). Since then, MRSA has developed into a worldwide public health problem and a particularly notorious pathogen (Delencastre *et al.*, 2007; Chambers, 1988). In 1999, it was reported that approximately 20%–25% of *S. aureus* isolated from patients in the US were resistant to methicillin (Herwaldt, 1999). By 2003, 64% of *S. aureus* infections in US intensive care units were thought to be caused by methicillin-resistant strains (Kleven et al., 2006). These statistics are especially alarming, as they reflect not only an increased incidence of resistant infections in hospitals, but also an increased risk to weakened and immunocompromized patients and a burden for the hospital system, as MRSA infections usually lead to prolonged stays in hospitals and increased costs of treatments and mortality (Cosgrove *et al.*, 2003 and 2005; Engemann *et al.*, 2003). Specifically, Cosgrove *et al.* (2005) estimated that average marginal additional costs resulting from MRSA infection totaled $3,836 per individual, while the Association of Professionals in Infection Control and Epidemiology estimate that the total cost of one MRSA infection approaches $30,000. It has also been
shown that the percentage of *S. aureus* isolates that are also MRSA increases with number of beds available for patients in the hospital, which further increases the risk of infection. For example, one study reported that hospitals with less than 200 beds, 200 to 499 beds, and greater than 500 beds, have 14.9%, 20.3% and 38.3% of *S. aureus* isolates identified as MRSA, respectively (Panlilio *et al.*, 1992).

Although MRSA infections have been historically associated with the healthcare environment (“healthcare-associated MRSA” (HA-MRSA)), the past 20 years have witnessed a change in the epidemiology of MRSA (Chambers, 2001, Kluymans-VandenBergh and Kluymans, 2006). During the past two decades, an increase in MRSA infections has been attributed to sources in the non-healthcare environment (i.e. the “community”), affecting individuals who exhibit none of the traditional risk factors for MRSA infection (i.e. exposure to healthcare). These isolates were termed community-associated MRSA (CA-MRSA) (CDC, 1999; Salgado *et al.*, 2003; Millar *et al.*, 2007) and were shown to infect a different population of people than that of HA-MRSA. For example, outbreaks of skin infections associated with CA-MRSA have been reported in prisons (Los Angeles, San Francisco, Mississippi, Georgia, and Texas), men who have sex with men, professional and collegiate athletes (e.g. football, wrestling, fencing), military, and certain native populations (Chambers, 2005; Kazakova *et al.*, 2005; CDC, 2003; Weber, 2005). CA-MRSA causes mild to severe infections, most often as skin and soft tissue infections (e.g. abscesses, cellulitis, folliculitis and impetigo), and it can also lead to hospitalization and/or death (Kluymans-Vanden Bergh and Kluymans, 2006). For example, Crum (2005) reported a fatality rate of 64% resulting from severe CA-MRSA infections, while 40% of the surviving individuals suffered from “significant
disabilities” afterwards, which further highlights the health risks associated with this pathogen.

The spread of CA-MRSA into the healthcare environment is attributed to MRSA carriage by asymptomatic or ailing individuals seeking in-hospital treatment for an infection or other medical purposes (Carleton et al., 2004), which subsequently increase the risk of infection in already susceptible patients in the hospitals (Carleton et al., 2004). For example, Purcell et al. (2005) reported that CA-MRSA infections constituted 98% of the MRSA infections in a children’s hospital in southern Texas, suggesting that CA-MRSA was spreading to the hospitalized population. CA-MRSA is now thought to account for 20% and 28% of all nosocomial and health-care bloodstream infections acquired in hospitals (Seybold et al., 2006).

Although reservoirs of CA-MRSA in the community include humans, inanimate shared items and domesticated animals, many community (non-hospital) environments remain poorly characterized in terms of their potential role as reservoirs of MRSA and their contribution to the spread of disease (Chambers, 2001; Weber, 2005; Stepanović et al., 2008). Recently, pets (cats and dogs), domesticated animals and veterinary clinics have been shown to harbor MRSA, suggesting the possibility of zoonotic transmission of MRSA strains (Huijsdens et al., 2006; Weese, 2004; Weese et al., 2005; van Loo et al., 2007; Leonard and Markey, 2008). The role of animals as a potential source of *Staphylococcus* infections was suggested long ago (Mann, 1959), and later reports established CA-MRSA as an emerging cause of zoonotic and veterinary disease (Huijsdens et al., 2006; Weese et al., 2005; van Loo et al., 2007; Leonard and Markey, 2008).
**Comparison between HA-MRSA and CA-MRSA:**

Although differences between CA-MRSA and HA-MRSA can be determined genetically, differences in the epidemiology (Table 1.3) are becoming less clear due to the spread of CA-MRSA into hospitals (in certain cases essentially replacing HA-MRSA) (Turnidge and Bell, 2000) and the potential transmission of HA-MRSA into the community (Chambers, 2001; Nathwani *et al.*, 2008; Seybold *et al.*, 2006; Gonzalez *et al.*, 2006). Interestingly, while HA-MRSA strains are usually resistant to different classes of antibiotics and carry a variety of antibiotic resistant genes, CA-MRSA often exhibits resistance that is mostly limited to the β-lactam class of antibiotics (Okuma *et al.*, 2002; Herold *et al.*, 1998). This limited susceptibility range of CA-MRSA is attributed to lower antibiotic pressure in a community setting as compared to that encountered in a hospital (Chambers, 2001). Conversely, the greater demand of broad-range antibiotic resistance has been shown to result in comparably lower fitness of HA-MRSA in the community setting (Chambers, 2001). However, it has been predicted that the success CA-MRSA in hospitals could facilitate the selection for strains that are additionally resistant to non-β-lactam antibiotics and adequately fit to proliferate in the community (Carleton *et al.*, 2004). Recently, Gonzalez *et al.* (2006) reported an increase in the scope of antibiotic resistance in CA-MRSA proliferating in hospitals, which appears to support the earlier prediction of Carelton *et al.*
Table 1.3: Comparison between HA-MRSA and CA-MRSA from Nathwani *et al.* (2008).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HA-MRSA</th>
<th>CA-MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical Patient</td>
<td>Elderly, immunocompromised and/or critically or chronically ill</td>
<td>Young, and healthy people</td>
</tr>
<tr>
<td>Infection</td>
<td>Bacteremia, pneumonia, and infections of wounds, ulcers and those associated with invasive surgery</td>
<td>Skin and soft tissue, necrotizing pneumonia, septic shock, and bone and joint infections</td>
</tr>
<tr>
<td>Transmission</td>
<td>Within healthcare setting and households in contact with the healthcare setting</td>
<td>Community (non-clinical) setting</td>
</tr>
<tr>
<td>Setting of Diagnosis</td>
<td>Inpatient settings and primary care</td>
<td>Outpatient or community setting</td>
</tr>
<tr>
<td>Patient’s Medical History</td>
<td>History of MRSA colonization, infection, surgery, invasive procedures and indwelling catheters, admission to healthcare settings, and exposure to antibiotics</td>
<td>No significant history or healthcare contact</td>
</tr>
<tr>
<td>Spread in Community</td>
<td>Limited</td>
<td>Spread occurs easily</td>
</tr>
<tr>
<td>Antibiotic Susceptibility</td>
<td>Often multi-resistant and choice of antibiotic therapy is of often very limited</td>
<td>Generally susceptible to a greater number of antibiotics, as compared to HA-MRSA</td>
</tr>
</tbody>
</table>

Differentiating between CA-MRSA and HA-MRSA is important clinically in order to prescribe appropriate therapy to infected patients and to minimize the spread of infections (Naimi *et al.*, 2003; Gonzales *et al.*, 2006). Since the spread of CA-MRSA and HA-MRSA have resulted in diffusion of MRSA epidemiology and a loss of outward characteristics that have traditionally been used to define CA- and HA-MRSA (Chambers, 2001), differentiation has been commonly achieved through genetic analysis, most often of the genes associated with methicillin resistance and toxin production. Specifically, HA-MRSA and CA-MRSA harbor the staphylococcal chromosomal cassette *mec* (SCCmec) that carries the gene (*mecA*), which confers resistance to the β-lactam class of antibiotics (Hanssen and Ericson Sollid, 2006 and others). Five major types of
SCCmec exist (SCCmec I-V) based on its genetic composition and the characteristics of recombinase genes associated with the SCCmec (Table 1.4) (Ito et al., 2001, 2004; Ma et al., 2002; Hanssen and Ericson Sollid, 2006; Martins and Cunh, 2007). The majority of HA-MRSA strains carry the SCCmec I (approximately 34 kb in size), II (53 kb), and III (67 kb) types, while SCCmec IV (21 to 24 kb) and V (28 kb) are the dominant types in CA-MRSA (Okuma et al., 2002; Ito et al., 2001 and 2003; Enright et al., 2002; Ma et al., 2002). SCCmec types IV and V are smaller than types I, II and III (Kluytmans-Vanden Bergh and Kluytmans, 2006) and lack antibiotic resistance genes other than mecA, which explains why resistance in CA-MRSA is mostly limited to the β-lactam class of antibiotics (Fey et al., 2003). However, the size of SCCmec IV and V can allow for increased mobility of the cassettes and relatively higher fitness of the CA-MRSA strains, which facilitates the efficient spread and persistence of this pathogen in the community (Robinson and Enright, 2003; Okuma et al., 2002; Ma et al., 2002; Oliveira et al., 2002).

Further genetic differences involve the detection of the Panton–Valentine leukocidin gene complex (PVL), which produces toxins that cause necrosis and leukocyte breakdown. The occurrence of PVL is usually indicative of CA-MRSA, while the complex rarely occurs in HA-MRSA (Vandenesch et al., 2003; Naimi et al., 2003) (Table 1.4). Additionally, typing methods such as multilocus sequence typing (MLST) (Enright et al., 2000 and 2002) and pulse-field gel electrophoresis (PFGE) (Chung et al., 2000) can be used to classify MRSA strains into HA-MRSA or CA-MRSA (Fig. 1.2).
Table 1.4: Comparison between the SCCmec types associated with HA-MRSA and CA-MRSA from Kluytmans-VandenBergh and Kluytmans (2006).

<table>
<thead>
<tr>
<th>SCCmec Type</th>
<th>Size (Kb)</th>
<th>Presence of other antibiotic resistance genes</th>
<th>Origin of isolates</th>
<th>Presence of PVL genes</th>
<th>Mean doubling time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>34</td>
<td>No</td>
<td>Hospital</td>
<td>Infrequent</td>
<td>36</td>
</tr>
<tr>
<td>II</td>
<td>53</td>
<td>Yes</td>
<td>Hospital</td>
<td>Infrequent</td>
<td>32</td>
</tr>
<tr>
<td>III</td>
<td>67</td>
<td>Yes</td>
<td>Hospital</td>
<td>Infrequent</td>
<td>42</td>
</tr>
<tr>
<td>IV</td>
<td>21-24</td>
<td>No</td>
<td>Community</td>
<td>Frequent</td>
<td>28</td>
</tr>
<tr>
<td>V</td>
<td>28</td>
<td>No</td>
<td>Community</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Figure 1.2: Genotypes of MRSA isolated from community versus hospital strains generated using pulse-field gel electrophoresis (Kazakova et al., 2005).
Coagulase-negative staphylococci (CNS)

Biochemically, staphylococci are broadly divided into two major groups based on their capacity to produce the coagulase enzyme (Martins and Cunha, 2007), which causes clotting of blood plasma. Coagulase-positive staphylococci include *S. aureus*, *S. hyicus*, and *S. intermedius* (Roberson *et al.*, 1992), while coagulase-negative staphylococci (CNS) compromise a more diverse collection of the other known staphylococci (Kloos and Bannerman, 1994). Like *S. aureus*, the CNS are part of the normal flora associated with humans and animals, primarily inhabiting the skin and mucous membranes (up to $10^4$-$10^6$ cells per cm$^2$) (Kloos and Bannerman, 1994; Noble and Pitcher, 1978). *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. cohnii*, *S. xylosus*, *S. capitis*, *S. warneri*, *S. hominis*, *S. simulans*, *S. saccharolyticus*, *S. auricularis*, *S. caprae*, *S. lugdunensis*, and *S. schleiferi* constitute the CNS commonly isolated from humans (Kloos and Bannerman, 1994). Interestingly, certain CNS species usually prefer specific niches in/on the human body. For example, *S. capitis* (*capit*: “head” in Latin) is mostly identified on the head, while *S. auricularis* (*auricul*: “ear” in Latin) is associated with the external ear canal (von Eiff *et al.*, 2002).

Historically, CNS were regarded as non-pathogens because of their inability to clot blood (Huebner and Goldmann, 1999). However, CNS were later found to be capable of causing serious infections and death in patients, especially those who were immunocompromised or undergoing invasive surgery and catheterization (Martins and Cunha, 2007). For example, CNS are responsible for 37% of all bloodstream infections in the US (Marshall *et al.*, 1998). As with *S. aureus*, the prevalence of antibiotic-resistant CNS is increasing (Marshall *et al.*, 1998; Martins and Cunha, 2007). This, combined
with their affinity for the materials used in indwelling medical devices (e.g. prosthetics, intravascular catheters) has contributed to their success as opportunistic, nosocomial pathogens (Huebner and Goldmann, 1999). Furthermore, CNS can cause a wide range of other infections including endocarditis, urinary tract infections, central nervous system shunt infections, endophthalmitis, surgical site infections and peritonitis (Piette and Verschraegen, 2008 and references therein). Today, CNS are considered equal or more important than \textit{S. aureus} in infections involving medical devices (Huebner and Goldmann, 1999). For example, 37.7\% of the isolates from bloodstream infection of intensive care unit patients were identified as CNS, while 12.6\% were identified as \textit{S. aureus} (National Nosocomial Infections Surveillance (NNIS), 1999).

\textit{S. epidermidis} is the most frequently isolated CNS (Martins and Cunha, 2007), followed by \textit{S. haemolyticus}, \textit{S. saprophyticus} and \textit{S. lugdunensis}, respectively (Heikens \textit{et al.}, 2005). Specifically, 65\% to 90\% of the staphylococci occurring in human samples were identified as \textit{S. epidermidis}, which inhabits various body locations including mucous membranes, and moist (groin, the armpit) and dry skin (Huebner and Goldmann, 1999). In recent years, \textit{S. epidermidis} has gained recognition as an opportunistic pathogen, affecting vulnerable patients in healthcare settings (Vuong and Otto, 2002). \textit{S. epidermidis} can cause a wide range of health problems, including bloodstream (bacteremia), throat, nose, ear, eye, cardiovascular (e.g. prosthetic-valve endocarditis and intravascular catheter infections), surgical wound, central nervous system, dialysis-associated and prosthetic-joint infections (Vuong and Otto, 2002; Heikens \textit{et al.}, 2005 and references therein). Unlike \textit{S. aureus}, \textit{S. epidermidis} produces few toxins that cause severe tissue damage, resulting in mostly chronic and relatively less-severe infections.
(Vuong and Otto, 2002; von Eiff et al., 2002). *S. epidermidis* can attach either directly to surfaces of indwelling medical devices or indirectly by attaching to proteins deposited on the device by the patient. The bacterium can then form a biofilm (a multilayered conglomerate of bacterial cells covered by a protective layer of extracellular material (slime)) (Fig. 1.3) that offers protection against antibiotics and host immune defenses (Vuong and Otto, 2002; von Eiff et al., 2002) and is considered a major virulence factor (Raad et al., 1998).

Like *S. aureus*, *S. epidermidis* can also exhibit resistance to antibiotics, including methicillin (Sawant et al., 2008). It was reported that 80% of healthcare *S. epidermidis* isolates were resistant to methicillin and many were resistant to additional antibiotics (Rupp and Archer, 1994).

![Figure 1.3: S. epidermidis colonizing a surface and forming a biofilm, multilayer of cells covered by slime (extracellular material) from von Eiff et al. (2002).](image)

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In addition to their increasing importance as pathogens, CNS are significant factors in the spread and development of antibiotic resistance in clinically important staphylococci (Hanssen and Ericson Sollid, 2006; Piette and Verschraegen, 2008), including community strains (Ruppé et al., 2008), largely as a result of the transfer of antibiotic-resistance genes to previously susceptible strains (Jaffe et al., 1980; Hurdle et al., 2005). In general, the frequency of methicillin resistance in CNS is higher than that observed in S. aureus isolates. For example, a study of bloodstream infections reported that 68% of CNS isolates were resistant to oxacillin (the antibiotic that replaced methicillin for the identification of methicillin resistance in staphylococci) vs. 26% of S. aureus isolates (Marshall et al., 1998). Most importantly, CNS constitute a major reservoir for mecA, which can be transferred from CNS to S. aureus through lateral gene transfer, giving rise to MRSA (Robinson and Enright, 2004; Hanssen and Ericson Sollid, 2006; Martins and Cunha, 2007). The acquisition of SCCmec by staphylococci, in general, and S. aureus, in particular, is considered the most important event in the evolution and spread of methicillin-resistant staphylococci (Hanssen and Ericson Sollid, 2006; Chambers, 2001; Delencastre et al., 2007; Foster, 2004).

The potential transfer of genetic material between CNS and S. aureus is not limited to mecA, but also includes plasmids that carry genes conferring resistance to different antibiotics, potentially increasing the spectrum of resistance in S. aureus or other recipient staphylococcal strains (Cookson, 1998; Hurdle et al., 2005; Elmerdahl Olsen et al., 2006; Martins and Cunha, 2007). For example, the blaZ gene, which can be carried on chromosome or plasmid and confers resistance to penicillins, can be
transferred between CNS and S. aureus (Elmerdahl Olsen et al., 2006). Plasmids conferring resistance to gentamicin were shown to transfer between S. eipdermidis and S. aureus (Jaffe et al., 1980). Furthermore, a conjugative plasmid carrying a gene responsible for high-level resistance to mupirocin (mupA) can transfer between CNS species (Udo et al., 1997), from CNS to S. aureus (Udo et al., 1997), and from S. epidermidis to MRSA (Hurdle et al., 2005).

The importance of CNS as agents of disease and the established relationship of CNS with MRSA highlights the need to avoid the historical bias of studying only one or a few staphylococci of interest. Rather, the staphylococci as a group, should be characterized, as many researchers are now suggesting that infection control measures should include CNS as well as MRSA (Piette and Verschraegen, 2008 and references therein). A shift in the approach to staphylococci research is increasingly necessary for understanding the evolution of antibiotic-resistance in these community and nosocomial pathogens, limiting the spread of infections, and enhancing decontamination and therapy efforts.

**Staphylococci in the non-hospital environment**

The study of staphylococci in the community has historically focused on health care facilities and specific at-risk populations outside hospital settings (Chambers, 1997). However, staphylococci have been isolated from the fecal material of birds and from the nasal cavities and other bodily parts of humans and domesticated animals (Vengust et al., 2006; Lefebvre et al., 2006, Williams et al., 1998; Medema et al., 1997; Lévesque et al., 2000). As a result, constant dermal and fecal shedding from animal sources likely drives
the occurrence of staphylococci in environments including fresh- and salt-water, beach sands, and wastewater effluents (Papadakis et al., 1997; Gabutti et al., 2004; Diaper and Edwards, 1994; Porter et al., 1993). The occurrence of staphylococci in some of these environments has created a risk for nonclinical infections. For example, outdoor swimming is a risk factor for *S. saprophyticus* infections of the urinary tract (Gatermann and Crossley, 1997; von Eiff et al., 2002). Furthermore, certain epidemic MRSA strains were reported to survive up to at least 14 days in river water and salt-water microcosms (Tolba et al., 2007).

Besides humans, the most important community reservoirs of staphylococci, including MRSA, are pets and livestock (van Duijkeren et al., 2004; Leonard and Markey, 2008). MRSA has been isolated from pigs (Lee, 2006; Von loo, 2007), horses (Weese, 2004; Weese et al., 2005), dogs, cats (Sing et al., 2008; Weese, 2004; Weese et al., 2005, van Duijkeren et al., 2005; Rankin et al., 2005), cattle, chicken (Lee, 2006), sheep (Goni et al., 2004), chinchillas, bats, and parrots (Morgan, 2008). In many cases, MRSA clones from animals were shared by their owners and/or handlers, suggesting the possibility for MRSA transmission between animals and humans (Weese, 2004; Weese et al., 2005; Sing et al., 2008). For example, Bradshaw (2003) provided evidence that MRSA could be transmitted from dogs to humans, while Sing et al. (2008) reported that similar MRSA isolates were collected from a cat and infected owner, therefore implicating the cat as a potential source of the pathogen. Recent evidence collected from SCCmec typing showed that MRSA isolates retrieved from companion animals were identical to epidemic strains occurring in hospitals (Leonard and Markey, 2008). In addition, MRSA was shown to be potentially transmissible within a veterinary clinic
setting (Seguin et al., 1999; Weese et al., 2004; Weese et al., 2004; Middleton et al., 2005).

Although the majority of these reports focused primarily on MRSA, the potential for the occurrence and transmission of a complex assembly of methicillin-resistant staphylococci has not been evaluated. This is important, since staphylococci that were historically regarded as nonpathogenic (Thylefors et al., 1998) are now known to pose a high risk of infection and has emerged as significant clinical and community pathogens (von Eiff et al., 2006; Vengust et al., 2006). Other methicillin-resistant staphylococci (e.g. S. intermedius and S. sciuri), which primarily occur in animals (Rich and Roberts, 2004), have been suggested to be also potentially zoonotic (Vengust et al., 2006 and references therein; Loeffler et al., 2007; Chen et al., 2007). Overall, considering the increasing incidence of community Staphylococcus infections and the role of CNS as important reservoirs for genes that significantly contribute to the evolution of MRSA, studies to develop adequate methodology to identify reservoirs of S. aureus and other staphylococci are warranted.

**Staphylococci on inanimate surfaces**

Although staphylococci are major contaminants of surfaces in clinical settings (Kloos and Bannerman, 1994 and references therein; Guyot and Layer, 2006; Poultsides et al., 2008), these bacteria are also ubiquitous on non-clinical inanimate surfaces, including shared items in the community setting. For example, staphylococci have been isolated from surfaces in commercial airplanes (S. epidermidis and S. hominis) (McManus and Kelley, 2005) and from surfaces in households including kitchen and
bathroom sinks, faucet handles, drains, dish sponges, cloths and towels, countertops, tubs, trays on infant chairs, and pet food dishes (Scott *et al.*, 2008). Additional items include hand rails used in public transportations systems, hygiene items (towels, razor blades) and gear shared among professional athletes and prisoners (Stepanović *et al.*, 2008; Kirkland and Adams, 2008; Kurkowski, 2007; Nguyen *et al.*, 2005; Begier *et al.*, 2004; CDC, 2003 and 2001). MRSA, specifically, has been isolated from hospital surfaces, including computers, bed rails, countertops, floors, door handles, faucets, bed linens, tables, blood pressure cuffs/tourniquets and gowns and gloves of healthcare personnel (Boyce *et al.*, 1997; Blythe *et al.*, 1998; Bures *et al.*, 2000; Devine *et al.*, 2001; Neely *et al.*, 2005; Wilson *et al.*, 2006; Snyder *et al.*, 2008).

The contamination of inanimate surfaces by staphylococci is probably driven by several factors, which include:

i- the ability of staphylococci to attach to surfaces (e.g. biofilm formation) (von Eiff *et al.*, 2002),

ii- the ubiquity of staphylococci in animal and human hosts and their shedding/transmission to surfaces (Martins and Cunha, 2007; Kloos and Bannerman, 1994; Guyot and Layer, 2006; Boyce, 2007),

iii- the absence of awareness of proper hygiene and/or decontamination (Scott and Bloomfield, 1990; Neely and Maley, 2000; Guyot and Layer, 2006),
iv- the type of material that constitutes the surface (Neely and Maley, 2000; von Eiff et al., 2002); and,

v- environmental conditions that affect the surface (e.g. presence of organic material, exposure to light, etc.) (Vuong and Otto, 2002; Sheldon et al., 2005).

Understanding the composition of the community surviving on contaminated surfaces is important, as survival of staphylococci on surfaces can promote the spread of these pathogens to patients or susceptible individuals in the community, increasing the risk of infection (Neely and Maley, 2000). *S. aureus* has been shown to survive on stainless steel and wood surfaces for as long as four days (Kusumaningrum et al., 2003) and on wood and vinyl for as long as three months (Makison and Swan, 2006). MRSA can persist for seven days to seven months on inanimate surfaces (Neely and Maley, 2000; Kramer et al., 2006; Makison and Swan, 2006), while methicillin-resistant CNS survived between 14-90 days on different types of fabrics encountered in the hospital (Neely and Maley, 2000). Interestingly, it has been reported that carriage of antibiotic resistance does not seem to affect the survival of these pathogens on surfaces (Neely and Maley, 2000; Duckworth and Jordens, 1990). In fact, one epidemic MRSA strain survived longer on dust than methicillin-sensitive *S. aureus* (Wagenvoort and Penders, 1997).

Surprisingly, little effort has been dedicated to identify the role of inanimate surfaces as reservoirs harboring an assembly of staphylococcal pathogens, determine the prevalence of different clinically-important staphylococcal species that might colonize
the surfaces and assess their potential transmission via surfaces in the nonhospital settings (Pancholi et al., 2005; Stepanović et al., 2008).

The SCCmec element and the origin and distribution of the mecA gene

The acquisition of the mobile genetic element, SCCmec, carrying a functional mecA gene marked the beginning of methicillin resistance in staphylococci (Ibrahem et al., 2009 and references therein; Leonard and Markey, 2008). The mecA gene encodes a protein called penicillin-binding protein (PBP; also known as PBP2’ and PBP2a), which functions as a transpeptidase catalyzing the cross-linking of peptidoglycan in the bacterial cell wall (Hartman and Tomasz, 1981; Berger-Bachi and Rohrer, 2002; Hanssen and Sollid, 2006). In antibiotic-sensitive strains, PBPs are bound and inactivated by β-lactam antibiotics (include penicillins, cephalosporins, and carbapenems), resulting in limited cell wall synthesis. However, PBP2a exhibits low binding affinity to β-lactam antibiotics allowing the continuation of cell wall biosynthesis and cell survival (Pinho et al., 2001).

SCCmec containing mecA is widely distributed in staphylococci and transmitted through lateral gene transfer between the species of this genus (Hiramatsu et al., 2001; Hanssen and Sollid, 2006). The precise transfer mechanism of SCCmec between staphylococci is unknown (Chambers, 2001, Ibrahem et al., 2009). However, SCCmec harbors, in addition to the mecA gene, mec regulatory genes and recombinase genes (cassette chromosome recombinase genes A and B, ccrAB ) (Leonard and Markey, 2008), which express DNA recombinase homologues that might be involved in the mobilization of SCCmec (Katayama et al., 2000). Specifically, in S. aureus the recombinase proteins
are implicated in catalyzing the excision, site-specific integration and orientation of the SCCmec in the chromosome (Chambers, 2001 and references therein).

Although the transfer of SCCmec between species is generally thought to be rare, suitable conditions (e.g. antibiotic pressure and increase in the abundance of methicillin-resistant staphylococci) that promote this transfer have been increasing (Chambers, 2001). Recent typing studies showed that the acquisition of SCCmec elements by epidemic methicillin-sensitive \textit{S. aureus} is thought to have had occurred on at least 20 separate occasions (Feil and Enright, 2004).

The origins of SCCmec are unknown, and it has not been detected in nonstaphylococcal bacteria (Hiramatsu \textit{et al.}, 2001). However, the \textit{mecA} sequence is highly conserved in \textit{Staphylococcus} species (Chambers, 1987; Ubukata \textit{et al.}, 1990; Archer \textit{et al.}, 1994) and is more widespread in individual CNS species than in \textit{S. aureus} strains (van Duijkeren \textit{et al.}, 2004). It is suggested that CNS have acquired \textit{mec} and recombinase genes from unknown sources before SCCmec was transferred to \textit{S. aureus} (Wu \textit{et al.}, 1996; Ibrahim \textit{et al.}, 2009 and references therein). Furthermore, Wu \textit{et al.} (1996) showed that over 150 independent isolates of \textit{S. sciuri}, a CNS species, exhibited a positive hybridization with a \textit{mecA} probe. Sequence analysis of \textit{S. sciuri} DNA revealed 88\% similarity to \textit{mecA} harbored by methicillin-resistant -\textit{S. aureus} and -\textit{S. epidermidis} (Wu \textit{et al.}, 1996). However, most of the \textit{S. sciuri} isolates harboring the \textit{mecA} homologue were susceptible to methicillin and penicillin, which suggested that the \textit{mecA} homologue might carry some function in \textit{S. sciuri} that was not related to resistance to methicillin (Wu \textit{et al.}, 1996). However, when \textit{S. sciuri} was experimentally stepwise subjected to increasing antibiotic (methicillin) pressure, its resistance to methicillin drastically
increased, as a point mutation was generated in the promoter of the \textit{mecA} homologue increasing its expression (Wu \textit{et al.}, 2001). When the mutant gene was introduced into methicillin-susceptible \textit{S. aureus}, the resistance of the \textit{S. aureus} strain to methicillin increased significantly (Wu \textit{et al.}, 2001). Since \textit{S. sciuri} is considered to be the most primitive among the staphylococci (Couto \textit{et al.}, 1996), it is generally suggested that it harbors the evolutionary staphylococcal precursor of \textit{mecA} (Fitzgerald and Musser, 2003). Consequently, \textit{mecA} was historically considered to be a property of the \textit{Staphylococcus} genus.

The detection of a \textit{mecA} homologue in \textit{Enterococcus hirae} (el Kharroubi \textit{et al.}, 1991) has led to speculation that \textit{mecA} might occur in nonstaphylococcal genera (Fitzgerald and Musser, 2003). However, the distribution and origin of \textit{mecA} in nonstaphylococcal species remain unknown or at best speculative (el Kharroubi \textit{et al.}, 1991; Wu \textit{et al.}, 1996 and 2001; Hiramatsu \textit{et al.}, 2001; Fitzgerald and Musser, 2003). However, this is important, as establishing the occurrence of \textit{mecA} in nonstaphylococcal genera and understanding its distribution and the mechanism of its transfer among species and/or genera might facilitate current epidemiological studies, enhance measures to decrease \textit{mecA} reservoirs, and allow designing methods to control its expression and limit its spread among species.

\textbf{Research addressing the detection and identification of staphylococcal reservoirs}
The recent increase in bacterial infections (Allison et al., 2007) (including those caused by staphylococci (reviewed in Rolston et al., 2007)), the newly recognized pathogen status of many of the staphylococci (Rolston et al., 2007; von Eiff et al., 2006; Kloos and Bannerman, 1994), and the transfer of clinically important genetic material among the staphylococcal species prompting the rise of multi-antibiotic resistant strains, emphasize the need to investigate the occurrence of staphylococci in both community and hospital settings. Although the CDC has initiated active surveillance of CA-MRSA in different locations in the US (CDC, 2004), this has not been generalized to include other staphylococci. Non-host reservoirs of MRSA have been identified in the clinical settings. However, the environmental reservoirs (e.g. shared surfaces and natural environment) of staphylococci, including *S. aureus* and CNS, have not been thoroughly identified. Furthermore, studies related to the contamination of surfaces (clinical or environmental) with staphylococci have historically focused on one or several species that were considered clinically important. For example, while MRSA is an important clinical, and now community, pathogen, the exhaustive focus on MRSA has resulted in decreased attention to other staphylococci that have been shown to exhibit clinical significance.

To address this void in our knowledge of an important group of potentially pathogenic bacteria, this research was designed to:

i- identify public surfaces that harbor antibiotic-resistant bacteria communities, including several members of the staphylococci,
ii- show that *meca* (an important genetic determinate that confers resistance to several popular therapeutic antibiotics) is widely distributed in nonstaphylococcal pathogens inhabiting a secondary environment, and;

iii- demonstrate a newly developed methodology that allows the simultaneous detection and species identification of most of the clinically important staphylococci, and apply the method to investigate the contamination of clinical surfaces with staphylococci.
Chapter Two

Public computer surfaces are reservoirs for methicillin-resistant staphylococci


Abstract

The role of computer keyboards used by students of a metropolitan university as reservoirs of antibiotic-resistant staphylococci was determined. Putative methicillin (oxacillin)-resistant staphylococci isolates were identified from keyboard swabs following a combination of biochemical and genetic analyses. Of 24 keyboards surveyed, 17 were contaminated with staphylococci that grew in the presence of oxacillin (2mg l$^{-1}$). Methicillin (oxacillin)-resistant *Staphylococcus aureus* (MRSA), -*S. epidermidis* (MRSE) and -*S. hominis* (MRSH) were present on two, five and two keyboards, respectively, while all three staphylococci co-contaminated one keyboard. Furthermore, these were found to be part of a greater community of oxacillin-resistant bacteria. Combined with the broad user base common to public computers, the presence
of antibiotic-resistant staphylococci on keyboard surfaces might impact the transmission and prevalence of pathogens throughout the community.

**Introduction**

The prevalence of bacterial infections in humans is increasing (Eguia and Chambers, 2003; Weber, 2005) and has been shown to result in part from transmission of pathogens from the hospital setting to the community and *vice versa* (Eguia and Chambers, 2003; Hidron *et al*., 2005; Lescure *et al*., 2006). Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase negative staphylococci (MR-CNS) have emerged as a significant nosocomial infectious threat, prompting several studies that have identified reservoirs of methicillin-resistant staphylococci in the hospital setting (Boyce *et al*., 1997; Blythe *et al*., 1998). Specifically, MRSA has been isolated from hospital surfaces, including computers, bed rails, countertops, floors, door handles, faucets, bed linens, tables, blood pressure cuffs/tourniquets, and gowns and gloves of healthcare personnel (Boyce *et al*., 1997; Blythe *et al*., 1998; Bures *et al*., 2000; Devine *et al*., 2001; Neely *et al*., 2005; Wilson *et al*., 2006; Snyder *et al*., 2008). Additionally, staphylococci including MRSA and antibiotic-resistant CNS, are major contaminants of catheters, indwelling surgical devices and implants (Kloos and Bannerman, 1994 and references therein; Guyot and Layer, 2006; Poultides *et al*., 2008). Staphylococci might survive on inanimate surfaces for few days to several months (Neely and Maley, 2000; Kramer *et al*., 2006; Makison and Swan, 2006), which would facilitate the spread of infection in hospitals and the community by prolonging the possibility of exposure to these pathogens to carriers and
hosts. In contrast with the healthcare setting, relatively few studies have been dedicated to identify the role of inanimate surfaces as pathogen reservoirs or determine the environmental conditions that affect staphylococci colonization, transmission and infection via surfaces in community settings (Pancholi et al., 2005; Stepanović et al., 2008). These studies have resulted in the isolation of methicillin-resistant staphylococci from nonclinical items, which included the hand rails used in a public transportations systems (Stepanović et al., 2008), surfaces in airliners (McManus and Kelley, 2005), personal hygiene items, and gear shared among professional athletes and prisoners (Kirkland and Adams, 2008; Kurkowski, 2007; Nguyen et al., 2005; Begier et al., 2004; CDC, 2003 and 2001), suggesting the important role played by reservoirs outside of the healthcare setting.

Because of frequent dermal contact by numerous users, a reservoir of interest is computer keyboards, which have been shown to harbor MRSA in the hospital setting (Bures et al., 2000; Devine et al., 2001; Neely et al., 2005; Wilson et al., 2006). Of increasing concern, however, is the role as pathogen reservoirs of keyboards in the nonhospital environment. Because the asymptomatic carriage of MRSA in humans is increasing (Creech et al., 2005; Hidron et al., 2005) along with the occurrence of community-associated MRSA infections (Purcell and Fergie, 2005), it follows that the ubiquitous sharing of public computers by a broad user base might facilitate increased transmission and prevalence of MRSA throughout the community. Furthermore, CNS and S. aureus occurring in humans occupy overlapping niches (Huebner and Goldmann, 1999; Becker et al., 2006), adding to the possibility for the simultaneous transmission and
colonization of multiple *Staphylococcus* spp and highlighting the importance of studying these pathogens that occur on community surfaces as an assembly.

The objective of this study was to identify computer keyboard surfaces used in a metropolitan university as reservoirs for methicillin-resistant staphylococci, including MRSA. For this purpose, a multiplex-PCR protocol was developed that, along with confirmatory biochemical methods, allowed the identification and differentiation of MRSA, other methicillin-resistant staphylococci and staphylococci that lack the *mecA*, the gene that confers resistance to methicillin in staphylococci. Since initial surveys suggested that keyboard surfaces were contaminated not only with MRSA, but also with mixed assemblages of antibiotic-resistant staphylococci, I additionally investigated the prevalence and structural complexity of oxacillin-resistant bacterial communities. This objective was achieved using denaturing gradient gel electrophoresis (DGGE), a molecular fingerprinting technique that allows the characterization of the potential structure/ members of a bacterial assembly (Muyzer et al., 1993).

**Materials and Methods**

**Sample collection:**

The surfaces of 24 computer keyboards in three, open-access, student computer facilities were sampled for bacteria with culture collection and transport swabs (Fisher Scientific, Inc., Pittsburgh, PA, USA). This was performed during operating hours featuring normal student and staff traffic. A control, ‘field blank’ swab that was briefly exposed to the air in each computer facility, was also collected to account for airborne bacteria. To select for methicillin-resistant bacteria, swabs were incubated (48 h at 35°C,
with shaking) in tryptic soy broth (TSB) supplemented with oxacillin (2mg l⁻¹) (Jonas et al., 2002). Oxacillin has replaced methicillin as the current agent of choice for selecting bacteria that are resistant to penicillinase-stable penicillins. However, the designation ‘methicillin-resistant’ is still widely used and readily interchangeable with ‘oxacillin-resistant’ (CLSI, 2006).

Following incubation, an aliquot (100 ml) from each turbid culture was inoculated onto Baird-Parker agar (Remel Inc., Lenexa, KS, USA), a selective medium used to isolate putative Staphylococcus spp (Baird and Lee, 1995). Forty-five colonies exhibiting morphology common to Staphylococcus spp (convex, shiny, black colonies) were randomly selected from among all positive enrichments. None of the enrichments of field blank swabs resulted in visible growth.

**Biochemical tests:**

To further confirm the identity of the putative Staphylococcus spp isolated in this study, the catalase and coagulase tests were used. All Staphylococcus spp are catalase positive, while S. aureus (and two other species, S. hyicus, and S. intermedius) is also coagulase positive. To perform these tests, putative Staphylococcus spp were transferred from Baird-Parker agar to Muller-Hinton broth, and incubated with shaking at 35° C for 24 hrs. One hundred microliters of growing culture was transferred onto a sterile slide and mixed with 2 drops of 3% hydrogen peroxide. Catalase activity was indicated by the immediate formation of bubbles, as catalase degraded hydrogen peroxide into water and oxygen. The presence of coagulase activity was tested by transferring 100 µl of growing culture to a tube containing 0.5 ml of rabbit plasma fibrinogen, followed by incubation at
35° C. Coagulase activity was indicated by clotting within the plasma either immediately or shortly after incubation. Two type strains, *S. aureus* (ATCC 6538) and MRSA (SB-01), were used as positive controls in all biochemical analyses.

**DNA isolation and Polymerase Chain Reaction (PCR):**

DNA from each coagulase- and/or catalase-positive culture was isolated using the remainder of the enrichment in Mueller-Hinton broth. Two milliliters of each culture was centrifuged at 13,000 x g for 2 min. to pellet the bacteria. The supernatant was discarded and the pellet was suspended in 1 ml of sterile DNA extraction buffer (pH 8) (50 mmol L\(^{-1}\) NaCl, 50 mmol L\(^{-1}\) Tris-HCl (pH 7.6), 50 mmol L\(^{-1}\) ethylenediaminetetraacetic acid (EDTA), 5% sodium dodecyl sulfate (SDS)). The suspension and 0.5 ml of 0.1 mm glass beads (Biospec Products Inc., OK, USA) were added to a 2 ml microcentrifuge tube and shaken in a bead beater (Fastprep, Bio101, Thermosavant, MN, USA) for 30 s at 5.5 m s\(^{-1}\) (Sigler and Zeyer, 2004). DNA isolation was carried out using phenol (pH 8)/chloroform extraction (as described in Sambrook and David, 2001). Isolated DNA was quantified by measuring optical density at A\(_{260}\) and diluted with DNA-grade water to a concentration of 100 μg ml\(^{-1}\) before further use.

Multiplex-PCR analysis was used to identify MRSA, other methicillin-resistant staphylococci, and staphylococci that did not contain *mecA* and were potentially not resistant to methicillin (Fig. 2.1a). Specifically, the multiplex-PCR targeted the 16S rRNA gene of *Staphylococcus* spp (Mason et al., 2001), *femB* (Jonas et al., 2002) and *mecA* (Jonas et al., 2002) (Table 2.1). The targeted *femB* sequence is *S. aureus*-specific and encodes a protein crucial for peptidoglycan crosslinking, while *mecA* has been
established as a standard marker for confirming methicillin resistance in staphylococci (CLSI, 2006). Isolates that were PCR-positive for all three genes were identified as MRSA, while those that were positive for the 16S rRNA gene and the \textit{mecA} were identified as other methicillin-resistant staphylococci. The presence of a positive reaction for \textit{femB} alone indicated \textit{S. aureus} that were potentially not resistant to methicillin, and the presence of the 16S rRNA gene alone indicated other staphylococci that were potentially not resistant to methicillin. Since \textit{S. epidermidis} is commonly associated with the human skin and is the most frequently isolated \textit{Staphylococcus} species (Kloos and Bannerman, 1994 and references therein), the identity of isolates that were not identified as \textit{S. aureus} or MRSA following initial analyses was assessed using a duplex PCR analysis that targeted two portions of the 16S rRNA gene specific to (i) \textit{Staphylococcus} spp and (ii) \textit{S. epidermidis} (Table 2.1) (Martineau \textit{et al.}, 1996). Isolates that were positive for both genes were identified as \textit{S. epidermidis}, while those that were positive only for the 16S rRNA gene were identified as other staphylococci.
Figure 2.1: (a) Representative results of multiplex-PCR analysis targeting the 16S rRNA gene of *Staphylococcus* spp, *femB*, and *mecA*. The isolates represented were chosen from among five different computer keyboards. Legend: M, 100 bp DNA size ladder; lanes 1–3, *mecA_ S. aureus*; lanes 4, 5, MRSA; lanes 6–9 and 15–19, *mecA Staphylococcus* spp (later identified as MRSE as described in text); lanes 10–14, *mecA_ Staphylococcus* spp; lane 20, MRSA-positive DNA control; lane 21, *mecA* S. aureus-positive DNA control; lane 22, negative DNA control (no template). (b) DGGE fingerprint of the 16S rRNA gene isolated from bacterial cultures growing in media supplemented with oxacillin (2mg l⁻¹). Lanes marked ‘∗∗’ exhibited additional bands that were not diagnostic of *S. aureus*, *S. epidermidis* and *S. hominis* (see positive control lanes) indicating the presence of a potentially complex oxacillin-resistant bacterial community on each personal computer keyboard (PC-'XX').
Table 2.1: Sequences of PCR primers used in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>5’-Forward primer-3’</th>
<th>5’-Reverse Primer-3’</th>
<th>Target size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus</em></td>
<td>16S rRNA</td>
<td>CTTTGAGTTTCAACTTGCTGG</td>
<td>791 bp</td>
</tr>
<tr>
<td><em>femB</em></td>
<td>TTACAGACTTATACGCTCTAC</td>
<td>ATACAAATCCAGCAGCCTCT</td>
<td>651 bp</td>
</tr>
<tr>
<td><em>mecA</em></td>
<td>GTAGAAAATGACTGAAAGCTCCGATAA</td>
<td>CCAATTTCCACATTGTCGCGGTTCA</td>
<td>510 bp</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>16S rRNA 1</td>
<td>ATCAAAAGTTGCGGAAACCTTTTCA</td>
<td>124 bp</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>16S rRNA 2</td>
<td>TCTCTTTTAATTTCTACTCTCAATCCATAG</td>
<td>174 bp</td>
</tr>
</tbody>
</table>

**Genotyping using Repetitive-Sequence PCR (rep-PCR):**

rep-PCR fingerprints were generated for *S. aureus* using the primer RW3A (5’-TCGCTCAAAAACACGACACC-3’) as described by Del Vecchio *et al.* (1995). rep-PCR is used to generate DNA fingerprint profiles of isolates and allows for the comparison and differentiation of genotypes between bacterial strains (Rademaker *et al.*, 1998). Fingerprints were generated by gel electrophoresis of the PCR product in 1.5% genetic analysis grade agarose containing 0.5 μg ml⁻¹ ethidium bromide. Fingerprints were documented using the Gel Logic 200 Imaging System (Kodak) and analyzed to detect unique strains (genotypes) with GelCompar II software (Version 3.5, Applied Maths) as described in Tenover *et al.* (1995).

**Denaturing Gradient Gel Electrophoresis (PCR-DGGE):**

To generate DGGE fingerprints that characterize the community of methicillin-resistant bacteria, PCR analysis was performed on the DNA, targeting the V3 region of the 16S rRNA gene by combining 0.2 mmoles of forward (341f 5’-CCTACGGGAGGGCAGCAG-3’) and reverse primers (534r 5’-ATTACCGCGGCTGCTGG-3’) (Muyzer *et al.*, 1993), 1 μl of DNA template, 1× PCR
buffer (Fisher Scientific, PA, USA), 1.5 mmol L⁻¹ MgCl₂, 1.5 mg ml⁻¹ bovine serum albumin (BSA), 0.2 mmol L⁻¹ of each dNTP, 0.02 U μl⁻¹ of Taq polymerase, and DNA-grade water to a final volume of 50 μl. To facilitate the separation of PCR products during DGGE analysis, a GC-clamp (Muyzer et al., 1993) was attached to the 5’ end of the forward primer. All sets of reactions included a positive control (Escherichia coli DNA) and a negative control containing no DNA template. Prior to DGGE analysis, PCR products (5 μl) were screened for successful reactions by electrophoresis in 1% agarose gel stained with 0.5 μg ml⁻¹ ethidium bromide. DGGE analysis was carried out according to the method of Sigler et al. (2004) using a DCode Universal Mutation Detection System (Bio-Rad) in polyacrylamide gels containing a denaturant gradient of 40–55% (100% denaturant represents 7 M urea and 40% vol/vol formamide). PCR and DGGE analysis of the bacterial communities were replicated three times in separate analyses. To identify the samples that contained S. aureus, S. epidermidis and/or S. hominis, alignment of the fingerprint patterns was performed using GelCompar II software (Version 3.5, Applied Maths), with a band position tolerance of 1% and optimization of 3% (Sigler and Pasutti, 2006). The DGGE profiles representing the samples were then compared to those generated from pure isolates of S. aureus, S. epidermidis and S. hominis.

**Identification of non-S. aureus and -S. epidermidis isolates by DNA sequencing:**

To identify those isolates that were determined not to be S. aureus or S. epidermidis by biochemical and molecular analyses, DNA sequencing was performed. Briefly, DNA was subjected to PCR targeting the V3 region of the 16S rRNA gene as
described above, following which the PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). The DNA was commercially sequenced in both directions (MWG Biotech Inc., NC, USA) and sequences were deposited in the National Center for Biotechnology Information Genbank database (Genbank accession numbers DQ831715–DQ831721). The putative identity of the organism associated with each sequence was determined using the BLAST algorithm (Altschul et al., 1990).

**Results and Discussion**

Samples collected from 17 of the 24 computer keyboards exhibited turbid growth in oxacillin-containing TSB broth, indicating that the surfaces harbored methicillin-resistant bacteria. PCR analysis of 52 oxacillin-resistant bacteria isolates (Fig. 2.1a) revealed that five keyboards were contaminated with *S. aureus*, two of which contained MRSA (Table 2.2). Although typical risk factors that predispose keyboards of clinical computers to MRSA contamination (e.g. narrow user base with high potential exposure to MRSA) were not present in the current study, the proportion of keyboards that harbored MRSA was comparable to that reported for computers in clinical settings (Devine et al., 2001; Fellowes et al., 2006). Furthermore, rep-PCR fingerprints of the MRSA isolates (n=6) showed that certain profiles possessed two distinct bands when compared to others, which indicated the presence of two readily distinguishable genotypes (Fig. 2.2). Both genotypes were found to contaminate each of the two keyboards that were positive for MRSA. Humans are known to harbor clonal populations of MRSA (Leski et al., 1998) that correspond to the predominant isolates or lineages throughout a community (Feil, 2003). Consequently, the rep-PCR results suggest a unique host origin of each MRSA
type, indicating that the computers were contaminated by multiple users/hosts carrying different MRSA strains. The occurrence of different MRSA strains on the keyboards represents an increased infectious risk to users, as multiple, potentially infectious MRSA strains are available for transmission to human hosts. These findings not only highlight the role of nonclinical computers as MRSA reservoirs, but also raises public health concerns when considering the large number of public computers available in the community (for example Internet cafes, public libraries and university campuses).

Figure 2.2: Molecular fingerprints of bacteria associated with computer keyboards. rep-PCR fingerprints of four representative *S. aureus* isolates (lanes 1 and 4 show two different genotypes of MRSA; lanes 2 and 3 show mecA (-) *S. aureus*) (M, 100 bp DNA size ladder).

PCR and biochemical analyses revealed that ten keyboards were contaminated with *S. epidermidis*, 5 of which harbored mecA-containing, methicillin-resistant *S. epidermidis* (MRSE) (Table 2.2). Because *S. epidermidis* has been shown to comprise a
significant proportion of the coagulase-negative staphylococci associated with humans (Ben Saida et al., 2006), the isolation of the bacterium from the keyboards was not surprising. However, the increased virulence in \textit{S. epidermidis}, including the acquisition of methicillin resistance (Blum and Rodvold, 1987; Huebner and Goldmann, 1999), further reinforces the role of keyboards as important pathogen reservoirs.

Table 2.2: Summary of PCR-based detection of \textit{S. aureus}, \textit{S. epidermidis}, and \textit{S. hominis} (including oxacillin-resistant strains; MRSA, MRSE and MRSH) isolated from computer keyboards.

<table>
<thead>
<tr>
<th>Staphylococci strain</th>
<th>Gene</th>
<th>No. of computers colonized (of 24 swabbed)</th>
<th>No. of isolates (52 total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. 16S rRNA(^a)</td>
<td>SE 16S rRNA(^a)</td>
<td>femB</td>
</tr>
<tr>
<td>\textit{S. aureus}</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MRSA</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>\textit{S. epidermidis}</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MRSE(^b)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MRSH(^c)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+, positive detection of gene; -, negative detection of gene.

\(a\) \textit{Staphylococcus spp}-specific 16S rRNA marker.

\(b\) \textit{S. epidermidis}-specific 16S rRNA marker.

\(c\) Identity confirmed following DNA sequencing.

Three keyboards were co-contaminated with MRSA and MRSE, suggesting that the bacterial contamination of the keyboards was not limited to simple bacterial assemblages, but rather resistant communities of considerable complexity. We assessed this complexity by performing denaturing gradient gel electrophoresis (DGGE) (Muyzer \textit{et al.}, 1993) of DNA directly isolated from the oxacillin supplemented TSB cultures described above. DGGE is a popular method for characterizing the structure of microbial assemblages by exploiting sequence heterogeneity of target genes (Muyzer \textit{et al.}, 1993). In this study, DGGE fingerprints revealed the presence of complex, oxacillin resistant
bacterial communities on eight of the keyboards (Fig. 2.1b). This complexity was also confirmed by sequencing (192 bp of the 16S rRNA gene; Muyzer et al., 1993) the DNA of seven, oxacillin-resistant (mecA-positive) staphylococci colonies collected from two of the culture-positive keyboards. BLAST analysis of these sequences revealed 100% similarity to *S. hominis* and *S. epidermidis* (Table 2.2) (GenBank accession numbers DQ831715–DQ831721). These results support the conclusion that the oxacillin-resistant bacterial community contaminating individual keyboards was not limited to MRSA and/or MRSE but was complex and included, for example, *S. hominis* (MRSH), a known nosocomial pathogen (Chaveset et al., 2005).

To our knowledge, the isolation of a mixed assemblage of oxacillin-resistant pathogens, including MRSA, MRSE and MRSH, from public, nonhospital fomites, has not been previously reported. The significance of this finding concerns not only the isolation of pathogens from public computer keyboards, but also the potential of transmission of antibiotic resistant pathogens to numerous computer users. The frequent occurrence of *mecA* in isolated staphylococci (Table 2.2) combined with the complexity of the bacterial community contaminating the keyboards (Fig. 2.1b) also raises concerns surrounding the potential for interspecies transfer of genes that confer antibiotic resistance. This has been shown to occur among staphylococci, including *S. aureus* and *S. epidermidis* (Forbes and Schaberg, 1983; Hanssen et al., 2004), and could effectively increase the diversity and number of antibiotic-resistant pathogens in this public reservoir. Of further importance, lateral gene transfer has been shown to occur in inanimate reservoirs. For example, an antibiotic susceptible *E. coli* strain on a towel
contaminated with milk successfully acquired a plasmid harboring resistance genes from a resistant strain (Kruse and Sorum, 1994).

Overall, our findings highlight the necessity for public awareness to hygiene following the use of public facilities and warrant further investigation into the epidemiology of community-associated MRSA and other staphylococci. The factors driving the contamination of the keyboards are unknown. However, the widespread nasal carriage of staphylococci by humans (Graham et al., 2006) likely facilitated the contamination via (i) hand-to-mouth or hand-to-nose contact while using the keyboard, and/or (ii) poor hand-washing habits (American Society for Microbiology, 2005). Furthermore, the contamination of inanimate surfaces by staphylococci is probably driven by several other factors, which include: (i) physiological and genetic disposition of staphylococci to attach to surfaces (e.g. biofilm formation) (von Eiff et al., 2002), (ii) the ubiquity of staphylococci in animal and human host and their shedding/transmission to the surfaces (Martins and Cunha, 2007; Kloos and Bannerman, 1994; Guyot and Layer, 2006; Boyce, 2007), (iii) the absence of awareness to proper hygiene and/or decontamination (Scott and Bloomfield, 1990; Neely and Maley, 2000; Guyot and Layer, 2006), (iv) the type of material that constitute the surface (Neely and Maley, 2000; von Eiff et al., 2002), and (v) environmental conditions that affect the surface (e.g. presence of organic material, exposure to light, etc.) (Vuong and Otto, 2002; Sheldon et al., 2005). Although we established that public computers are reservoirs of oxacillin-resistant pathogens, the duration of bacterial survival and transmission rate to humans in community settings remains unknown.
Surprisingly, little effort has been dedicated to identify the role of inanimate surfaces as pathogen reservoirs or to determine the environmental conditions that affect staphylococci colonization, transmission and infection via surfaces in the non-hospital settings (Pancholi et al., 2005; Stepanović et al., 2008). Therefore, subsequent steps to limit the spread of antibiotic resistant pathogens throughout the community should include efforts to not only increase awareness of appropriate hygiene and decontamination strategies, but also to elucidate the ecology of bacteria contaminating community surfaces.
Chapter 3

Occurrence of mecA in nonstaphylococcal pathogens in surface waters


Abstract

The rapid spread of antibiotic resistance determinants can potentially increase environmental reservoirs of multiple antibiotic-resistant bacteria, such as methicillin-resistant staphylococci. One antibiotic-resistance gene of concern is mecA, which confers resistance to a wide range of β-lactams in clinical and community strains of staphylococci. Since little is known about the prevalence of mecA in the environment and its occurrence in nonstaphylococcal genera, we assessed the presence of mecA in antibiotic-resistant bacteria inhabiting natural waters near Bay Village, Ohio. Water samples were collected from 13 sites and bacteria were isolated on Baird-Parker agar containing oxacillin. Five bacteria isolates (total = 65) were selected from each site and tested for the presence of mecA using PCR analysis. Randomly selected mecA-positive isolates representing all the sites in the study were identified by sequencing the 16S rRNA gene, genotyped using BOX-PCR, and assayed for resistance to different antibiotics. mecA was identified in 44 isolates representing three species of pathogenic bacteria, including Proteus vulgaris, Morganella morganii and Enterococcus faecalis.
Isolates of *P. vulgaris* exhibited six unique BOX-PCR fingerprints, as compared to one and two for *M. morganii* and *E. faecalis*, respectively. All isolates were resistant to oxacillin, ampicillin, amoxicillin, vancomycin, erythromycin, and tetracycline, while none were resistant to ciprofloxacin and trimethoprim. For the first time, we report the detection of *mecA* in three species of multiple antibiotic-resistant pathogens. The occurrence of *mecA* in these unrelated species suggests the potential for *mecA* exchange among environmental bacteria.

**Introduction**

The success of bacterial pathogens in the environment is driven by their ability to adapt, spread and establish ecological reservoirs (Hanssen and Ericson Sollid, 2006). An important determinant of this adaptation is the acquisition of genes that confer resistance, or increase already existing resistance, to antibiotics (Hanssen and Ericson Sollid, 2006). Antibiotic resistance allows bacterial pathogens to survive antibiotic pressure in both the healthcare settings and the natural environments. For example, while in 1961 methicillin-resistant strains of *S. aureus* were considered as rare laboratory curiosities (Chambers, 1988), in 1999 approximately 20%–25% of *S. aureus* isolated from patients in the U.S. were resistant to methicillin (Herwaldt, 1999). Recent interest has focused on the presence of antibiotics and other pharmaceuticals in natural waters and overland runoff (Spongberg and Witter, 2008), as the presence of these chemicals has been shown to select for highly-resistant bacteria in nonclinical settings (Kruse and Sorum, 1994). Additionally, public health concerns surround the increasing trend in human infections associated with non-healthcare settings (i.e. the community) (Martins and Cunha, 2007). For example, community-associated strains of methicillin-resistant *S. aureus* (MRSA)
account for 20% and 28% of all nosocomial and health care bloodstream infections (Seybold et al., 2006).

Pathogenic staphylococci are known to colonize the fecal material of birds and the nasal cavities and other body surfaces of humans and domesticated animals (Vengust et al., 2006; Lefebvre et al., 2006; Williams et al., 1998; Medema et al., 1997; Lèvesque et al., 2000). Staphylococci are also known to contaminate several natural environments including fresh- and salt-water, beach sands, and wastewater effluents (Papadakis et al., 1997; Gabutti et al., 2004; Diaper and Edwards, 1994; Porter et al., 1993). The occurrence of staphylococci in these nonclinical environments can represent an infectious risk. For example, outdoor swimming constitutes a risk factor for infections with S. saprophyticus, a pathogen of the urinary tract (Gatermann and Crossley, 1997; von Eiff et al., 2002). Although few data exist concerning the occurrence of methicillin-resistant S. aureus (MRSA) in natural waters, some epidemic MRSA strains can survive up to 14 days in river-water and salt-water microcosms (Tolba et al., 2007), raising further concern about the potential role of the water environment in maintaining and spreading these pathogens.

Despite the recognized pressures selecting for antibiotic resistance in environmental pathogens, there is little understanding of the role of the natural environment as a reservoir of staphylococci and other potentially pathogenic bacteria that might harbor important antibiotic resistance genes. Considerable recent attention has focused on methicillin-resistant staphylococci, as well as mecA, the gene that confers resistance to the β-lactam class of antibiotics, including methicillin. The mecA gene encodes a protein called penicillin-binding protein (PBP; also known as PBP2’ and
PBP2a), which functions as a transpeptidase, catalyzing the cross-linking of peptidoglycan in the bacterial cell-wall (Hartman and Tomasz, 1981; Berger-Bachi and Rohrer, 2002; Hanssen and Sollid, 2006). In antibiotic-sensitive strains, PBPs are bound and inactivated by β-lactam antibiotics (which include penicillins, cephalosporins, and carbapenems), resulting in limited cell-wall synthesis. However, PBP2a exhibits low binding affinity to β-lactam antibiotics allowing the continuation of cell-wall biosynthesis and cell survival (Pinho et al., 2001). In general, resistance genes are known to have a wide host range (Hanssen and Ericson Solli, 2006). In fact, although meca was reported to be most commonly associated with staphylococci (Hanssen et al., 2004), the detection of a meca sequence homologue in Enterococcus hirae (el Kharroubi et al., 1991) suggested that meca also occurs in nonstaphylococcal genera (Fitzgerald and Musser, 2003) and raised awareness that the hosts of this important gene are not limited to the staphylococci. However, there have been no more reports of meca homologues occurring in other bacteria species.

The acquisition of the mobile genetic element, SCCmec, containing meca marked the beginning of resistance to β-lactam antibiotics in staphylococci (Ibrahem et al., 2008 and references therein; Leonard and Markey, 2008). SCCmec carrying meca is known to be widely distributed in staphylococci and transmitted through lateral gene transfer between the species of this genus (Hiramatsu et al., 2001; Hanssen and Sollid, 2006). Although the transfer of SCCmec between species is generally thought to be rare, suitable conditions (e.g. antibiotic pressure and increase in the abundance of methicillin-resistant staphylococci, i.e. SCCmec DNA) that promote this transfer have been increasing (Chambers, 2001). However, the meca sequence was considered to be highly conserved
in *Staphylococcus* species (Chambers, 1987; Ubukata *et al.*, 1990; Archer *et al.*, 1994), and the distribution of *meca* in nonstaphylococcal species was not well characterized (el Kharroubi *et al.*, 1991; Wu *et al.*, 1996 and 2001; Hiramatsu *et al.*, 2001; Fitzgerald and Musser, 2003). Understanding the distribution of *meca* in nonstaphylococci and the factors affecting its persistence in the environment might enhance measures to decrease *meca* reservoirs, thereby limiting the infectious risk posed by potential-antibiotic-resistant pathogenic species. Therefore, I investigated in this study the occurrence of methicillin-resistant staphylococci and other *meca*-containing bacteria in surface waters that were impacted by fecal pollution and urban water runoff and have been implicated in outbreaks of bacterial disease, including gastroenteritis, dermatitis, and acute respiratory illness (Dorfman and Stoner, 2007).

Although methicillin-resistant staphylococci were not isolated from the waters that were investigated in this study, nonstaphylococcal *meca*-containing pathogens that included *Proteus vulgaris*, *Morganella morganii* and *E. faecalis* were detected. Consequently, the sequences of *meca* from these pathogens, their antibiotic-resistant properties, genetic diversity, and their occurrence in the water samples were investigated using different biochemical and molecular methods.

**Materials and Methods**

**Sample collection and processing:**

Thirteen grab water samples (1 L) were collected from Huntington Beach and Porter Creek (Bay Village, Ohio) (Fig. 3.1). Samples were put in ice and processed within 24 h of sampling. One hundred milliliters of each water sample was filtered
through a nitrocellulose filter membrane (0.45 µm pore size), following which the filters were transferred to tubes containing 15 ml of Muller-Hinton (MH) broth supplemented with 6 mg l⁻¹ of oxacillin and 25,000 IU of polymyxin. The tubes were incubated at 35º C for 16-18 h with shaking.

Figure 3.1: Satellite photograph showing the locations of the 13 sites along Porter Creek and Huntington Beach (Bay Village, Ohio) from which water was collected.

**Isolation of oxacillin-resistant bacteria:**

An inoculum (50 µl) from each sample that exhibited visible growth in MH broth supplemented with oxacillin was spread onto Baird-Parker agar supplemented with 6 mg l⁻¹ of oxacillin and 25,000 IU of polymyxin and incubated for 24 h. If needed, the plates were incubated for an additional 24 h to allow a full development of the *Staphylococcus*
bacteria phenotype. Five colonies from each plating (total of 65) that exhibited a *Staphylococcus* species phenotype (shiny black, convex) were randomly selected from each sample. Of the 65 selected isolates, sixty isolates were additionally surrounded by a halo, a diagnostic feature of *S. aureus* growth on Baird-Parker agar. The colonies were subjected to Gram staining and catalase and coagulase tests as described in Chapter 2. After the biochemical analysis, it was determined that some of the isolates might belong to the *Enterococcus* genus. Consequently, each colony was also transferred to two other selective media including Slantez and Bartley agar (Oxoid) and mEI agar (BD Diagnostics), both of which are diagnostic for *Enterococcus* species.

**DNA isolation:**

Selected colonies were individually enriched in Mueller-Hinton broth (MH), and DNA from the enrichments was isolated as follows. Two milliliters of each culture was transferred to a sterile 2 ml microcentrifuge tube and centrifuged at 13,000 x g for 2 min to pellet the bacteria. The supernatant was discarded and the pellet was suspended in 1 ml of sterile DNA extraction buffer (pH 8) (50 mmol L\(^{-1}\) NaCl, 50 mmol L\(^{-1}\) Tris-HCl (pH 7.6), 50 mmol L\(^{-1}\) ethylenediaminetetraacetic acid (EDTA), 5% sodium dodecyl sulfate (SDS)). Approximately 0.5 ml of glass beads (0.1 mm diameter; Biospec Products Inc., OK, USA) were added and followed by bead beating (Fastprep, Bio101, Thermosavant, MN, USA) for 30 s at 5.5 m s\(^{-1}\) (Sigler and Zeyer, 2004). DNA was purified using phenol (pH 8)/chloroform extraction as described in Sambrook and David (2001) and quantified by measuring optical density at \(A_{260}\). The purified DNA was diluted with DNA-grade water to a concentration of 100 μg ml\(^{-1}\) and stored at -20°C until further use.
Multiplex-PCR and P. vulgaris- and E. faecalis-Specific Polymerase Chain Reaction:

A multiplex PCR protocol was designed to detect methicillin-resistant staphylococci by targeting the 16S rRNA gene of Staphylococcus spp (Mason et al., 2001), femB (Jonas et al., 2002) and mecA (Jonas et al., 2002) (Table 3.1). The targeted femB sequence is S. aureus-specific, while mecA is an established standard marker for confirming methicillin resistance in staphylococci (Clinical and Laboratory Standards Institute, 2006). The multiplex-PCR was performed under the following conditions: 94 °C for 5 min followed by 40 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 45 sec, and 72 °C for 30 min.

Biochemical analyses (including Gram staining, the catalase test, and the API system described below) revealed that P. vulgaris and E. faecalis comprised the mecA-containing bacteria community. This putative identification was confirmed with (i) a P. vulgaris-specific PCR analysis targeting the urease gene (384 bp) (Table 3.1), which was performed as described by Kupfer et al. (1999) and (ii) an E. faecalis-specific PCR analysis targeting the ddl gene encoding D-alanine–D-alanine ligases (941 bp) (Table 3.1), which was performed as described by Kariyama et al. (2000). All reactions included two positive controls (P. vulgaris (ATCC 6896) and E. faecalis (ATCC 29212) DNA) and a negative control containing no DNA template. PCR products were analyzed by electrophoresis in 1% agarose gels (1.5% for the multiplex-PCR products) that were stained with 0.5 μg ml⁻¹ ethidium bromide. The size of the PCR product generated from the DNA of the unknown isolates and that of P. vulgaris (ATCC 6896) and E. faecalis
(ATCC 29212) was determined by comparison to a 100 bp DNA-ladder marker. Unknown isolates were identified as belonging to a species if the PCR from their DNA showed the species-specific gene signal of the expected base-pair size (384 bp for *P. vulgaris* and 941 bp for *E. faecalis*).

Table 3.1: Sequences of PCR primers used in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>5′-Forward primer-3′</th>
<th>5′-Reverse Primer-3′</th>
<th>Target size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus</em> 16S</td>
<td>CCTATAAGACTGGGAATACTTCCGGG</td>
<td>CTTTGAGTTTCAACCTTGCGGTCG</td>
<td>791 bp</td>
</tr>
<tr>
<td>rRNA</td>
<td>femB</td>
<td>ATACAAATCCAGCACAACCTCTT</td>
<td>651 bp</td>
</tr>
<tr>
<td>meca</td>
<td>GTAGAAATGACTGAAAGTGGCTGATAA</td>
<td>CCAATTCACATTTGTTTGGGTCTA</td>
<td>310 bp</td>
</tr>
<tr>
<td><em>S. epidermidis</em> 16S</td>
<td>ATCAAAAAAGTTGCGGAAACCTTTCA</td>
<td>CAAAAGAGCGGTGGAGAAAATTCATCA</td>
<td>124 bp</td>
</tr>
<tr>
<td>rRNA 1</td>
<td><em>S. epidermidis</em> 16S</td>
<td>TCTTTTTTAAATTCATTTCAATTCCATAG</td>
<td>174 bp</td>
</tr>
<tr>
<td>rRNA 2</td>
<td></td>
<td>AAACACAAATTCAAGCTGTTTCCCATATC</td>
<td></td>
</tr>
<tr>
<td>Bacterial 16S rRNA (Y3)</td>
<td>CCTCGCGAGCCAGCGAG</td>
<td>ATTACCGGCTGCTGG</td>
<td>192 bp</td>
</tr>
<tr>
<td>BOX A1R</td>
<td>CTAAGGAGCGGCTGACG</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>P. vulgaris</em> vrrA</td>
<td>GCTAAATACGCAGATACGTCG</td>
<td>AACCTGTCACCCCTCGGTA</td>
<td>384 bp</td>
</tr>
<tr>
<td>gene</td>
<td><em>E. faecalis</em> vrrA</td>
<td>TCAAGTACAGTTACTTCTTAGA</td>
<td>541 bp</td>
</tr>
</tbody>
</table>

**Antibiotic-resistance assays:**

All assays to determine antibiotic-resistance among the *mecA*-containing isolates were conducted as described previously (CLSI, 2006). Briefly, purified *mecA*-containing isolates were transferred to Muller-Hinton broth and enriched following incubation for 16-18 h. The cultures were diluted to 0.5 McFarland units (which was quantified by measuring optical density using a spectrophotometer and comparison to a 0.5 McFarland standard) and inoculated onto MH agar containing increasing concentrations (0 to 128 mg l⁻¹) of antibiotics, including oxacillin, ampicillin, and amoxicillin (β-lactam antibiotics); vancomycin (glycopeptide); erythromycin (macrolide); tetracycline (tetracycline); trimethoprim (folate pathway inhibitors), and ciprofloxacin (fluoroquinolones). The cultures were incubated at 35° C for 16-18 h. The minimum inhibitory concentration
(MIC) was determined for each isolate as the lowest concentration of antibiotic that completely inhibited bacteria growth. Pre-identified isolates of *P. vulgaris* (ATCC 6896), *M. morganii* (NRRL B-1663), *E. faecalis* (ATCC 29212) *S. aureus* (ATCC 6538), MRSA (SB-01), and *S. epidermidis* (ATCC 146) were used as positive-control organisms in all assays.

**Denaturing Gradient Gel Electrophoresis (DGGE):**

Total DNA isolated from the enrichments of the filtered water samples (see above) was subjected to PCR analysis, targeting the V3 region of the 16S rRNA gene by combining 0.2 mM each of forward and reverse) primers (Muyzer et al., 1993) (Table 3.1), 1 µl of DNA template, 1× PCR buffer (Fisher Scientific, PA, USA), 1.5 mmol L⁻¹ MgCl₂, 1.5 mg ml⁻¹ bovine serum albumin (BSA), 0.2 mmol L⁻¹ of each dNTP, 0.02 U µl⁻¹ of *Taq* polymerase, and DNA-grade water to a final volume of 50 µl. To facilitate the separation of PCR products during DGGE analysis, a GC-clamp (Muyzer et al., 1993) was attached to the 5’ end of the forward primer. All sets of reactions included DNA from a positive control organism (*Escherichia coli* DNA) and a negative control containing no template. PCR products (5 µl) were screened by electrophoresis in 1% agarose gel stained with 0.5 µg ml⁻¹ ethidium bromide. The PCR products were compared to a 100 bp DNA-ladder marker to determine if they exhibited the expected base-pair size (192 bp). DGGE analysis was carried out according to the method of Sigler et al. (2004) using a DCode Universal Mutation Detection System (Bio-Rad) in gels containing a denaturant gradient of 40–60% (100% denaturant represents 7 M urea and 40% vol/vol formamide). To determine if the query bacterium species (*S. aureus, P. vulgaris, E. faecalis,* and *M. morganii*) might be a part of the larger resistant community
comprising each sampled site, the DGGE bands representing these species were compared to the bands that constituted the community. The analysis of band positions in each fingerprint patterns was carried out using GelCompar II software (Version 3.5, Applied Maths), with a band position tolerance of 1% and optimization of 3% (Sigler and Pasutti, 2006).

**Identification of isolates by sequencing and biochemical methods:**

To identify those isolates that were determined not to be staphylococci by biochemical and molecular analyses described above, DNA sequencing was performed. Briefly, DNA was subjected to PCR analysis targeting the 16S rRNA gene (approximately 1000 bp) (Hugenholtz et al., 1998) as described above, following which the PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). The DNA was commercially sequenced in both directions (MWG Biotech Inc., NC, USA) and sequences were deposited in the National Center for Biotechnology Information Genbank database (Genbank accession numbers EU710760-EU710762; EU736118-EU736139 ). The putative identity of the organism associated with each sequence was determined using the BLAST algorithm (Altschul et al., 1990).

The identity of the sequenced isolates was further confirmed using the API 20E (for identification of *Enterobacteriaceae*) and 20 Strep (for identification of enterococci) systems (Biomerieux, USA) according to protocols supplied by the manufacturer. Briefly, for identification of enterococci with the API 20E system, freshly isolated colonies of the subject bacterium was homogenized in 5 ml of sterile distilled water and then transferred to each of the 20 wells on the test strip. A drop of sterile mineral oil was added to the test wells that required anaerobic conditions. The strips were incubated at
37°C for 18-24 h in a humidified container. Following incubation, chemical reagents were added to the test wells according to the manufacturer’s instructions. Color formation in the test wells was compared to a table supplied by the manufacturer, and each test reaction in the strip was considered either positive or negative. The results were translated into a 7-digit code as specified by the manufacturer and then compared to an identification table to determine the identity of the isolate. Similar steps were required for the API 20 Strep system. However, the original suspension was homogenized to 4.0 McFarland units, which was quantified by measuring optical density using a spectrophotometer and comparison to a 0.5 McFarland standard. Throughout the experiment, isolates of *P. vulgaris* (ATCC 6896), *M. morganii* (NRRL B-1663), *E. faecalis* (ATCC 29212) *S. aureus* (ATCC 6538), and *S. epidermidis* (ATCC 146) were used as positive-control organisms.

**Sequencing of mecA:**

To determine the relationship of the *mecA* sequences detected in nonstaphylococcal species to those commonly associated with methicillin-resistant staphylococci, DNA from *mecA*-containing isolates (n=25) was again subjected to PCR analysis targeting approximately 310 bp of the *mecA* (Jonas et al., 2002) as described above. The PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and commercially sequenced in both directions (MWG Biotech Inc. (NC, USA)). To determine if similarities existed between these sequences, the sequences were compared to *mecA* sequences from staphylococci deposited in Genbank using ClustalW and the BLAST algorithm (Altschul et al., 1990). All sequences were
deposited in the National Center for Biotechnology Information Genbank database (Genbank accession numbers EU710763-EU710764; EU736140).

**BOX-PCR analysis of mecA-containing isolates:**

BOX-PCR analysis was performed following the protocols of Rademaker et al. (1998) and using the BOX A1R primer (5’-CTACGGCAAGGCGACGCTGACG-3’) for each of the mecA-containing isolates to assess their genotypic similarity. Fingerprints resulting from BOX-PCR analysis were compared following electrophoresis in 1.5% agarose gels containing ethidium bromide along with a 100 bp DNA marker that was loaded into the gel such that marker lanes were separated by no more than five PCR sample lanes. Images of the fingerprints were archived using the Gel Logic 200 Imaging System (Kodak). Using cluster analysis in the GelCompar II software and the Pearson’s correlation coefficient for comparing the bands intensity and position, fingerprints from each species that exhibited greater than 90% similarity were considered belonging to the same genotype (Sigler and Pasutti, 2006).

**Results and Discussion**

All filtered water samples (n=13) resulted in bacterial growth when incubated in MH broth supplemented with oxacillin. Isolates enriched from each sample along with pre-identified control bacteria including MRSA (SB-01) (Tenover et al., 1994), *S. aureus* (ATCC 6538) and *S. epidermidis* (ATCC 146) exhibited typical staphylococcal growth phenotype (black, convex, shiny colonies, and a halo surrounding *S. aureus* colonies) on the Baird-Parker agar. However, multiplex PCR analysis showed that while mecA was detected in 44 of the 65 isolates that were selected from all 13 enrichments, none were
staphylococci (Fig. 3.2). The detection of mecA in nonstaphylococcal bacteria was unexpected, as mecA was considered to be commonly associated with the staphylococci (Hanssen et al., 2004) and essential in the expression of methicillin resistance in this genus (Hanssen and Sollid, 2006). Further attempts to identify the mecA-containing bacteria using DNA sequencing, API 20E and 20 Strep systems (Biomerieux, USA) and a series of other tests (Table 3.2) revealed that the isolates were Proteus vulgaris (n=18 of 25 isolates tested), Morganella morganii (n=4) and Enterococcus faecalis (n=3). P. vulgaris, M. morganii and E. faecalis are known clinical pathogens that are capable of causing human illness, including urinary-tract, wound, and respiratory infections (Drago et al., 2001; Falagas et al., 2006; Lau et al., 2004; Huycke et al., 1998). Additionally, these species are also known to occur in fresh-water environments and waters impacted with sewage, potentially causing pathological symptoms in humans and other organisms (Rózalski et al., 1997; Harwood et al., 2004 and references there in). Although Baird-Parker agar was originally designed to isolate staphylococci, the growth of these bacteria was not surprising, as each is known to tolerate the supplements used to select against non-lecithinase-producing bacteria (tellurite) (Toptchieva et al., 2003, Ruoff et al., 1990) and other Gram-negative species (polymyxin B) (Gales et al., 2006; Fabretti et al., 2006). This was further confirmed when pre-identified strains of P. vulgaris (ATCC 6896), M. morganii (NRRL B-1663) and E. faecalis (ATCC 29212) exhibited a phenotype on Baird-Parker agar similar to control strains of S. aureus (ATCC 6538) and S. epidermidis (ATCC 146).
Figure 3.2: PCR analysis of *mecA* detected in a representative sample of nonstaphylococcal isolates from surface waters. Legend: M, 100 bp DNA size ladder; *, nonstaphylococcal isolates that were *mecA* negative; MRSA, PCR of *mecA* from a MRSA-positive control; -, negative control. The lower bands are excess primer.

<table>
<thead>
<tr>
<th>Identity of <em>mecA</em>-containing isolates (n)</th>
<th>Biochemical test</th>
<th>Growth/appearance on diagnostic medium</th>
<th>Species-specific PCR analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram stain</td>
<td>Catalase</td>
<td>Swarming</td>
</tr>
<tr>
<td><em>P. vulgaris</em> (18)</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. morganii</em> (4)</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>E. faecalis</em> (3)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Preidentified strains of *Proteus vulgaris* (ATCC 6896), *Morganella morganii* (NRRL B-1663), and *Enterococcus faecalis* (ATCC 29212) were used as controls in each assay. +, positive result; -, negative result, +/–, intermediate result.

b n, no. of isolates.

* mEL (BD Diagnostics) and Slanetz and Bartley (Oxoid) media are differential and selective for *Enterococcus* spp.

d For *P. vulgaris*, see Kupfer et al. (1999); for *E. faecalis*, see Kariyama et al. (2000).

e Isolates of *M. morganella* exhibited a weak PCR signal.

The detection of *mecA* in the majority of the nonstaphylococcal colonies (n=44) isolated in this study showed that the distribution of the gene extends beyond the
staphylococci. Interestingly, it has been suggested that mecA originated from homologous sequences in Staphylococcus sciuri (regarded as the most primitive among the staphylococci (Couto et al., 1996)) or Enterococcus hirae, and then became established in species of clinically-significant staphylococci, including S. aureus and S. epidermidis (Hanssen et al., 2004; El Kharroubi et al., 1991; Fitzgerald and Musser, 2003). Consequently, it was necessary to establish the relationship between the mecA sequence detected in this study and the sequence commonly associated with methicillin-resistant staphylococci; i.e. the newly discovered sequence was tested to determine if it was closely related to those found in clinically important staphylococci or was a distant homologue. mecA DNA sequence alignment revealed that the newly identified mecA exhibited 100% similarity to sequences of mecA found in S. aureus and other staphylococci that harbored the staphylococcal cassette chromosome mec (SCCmec) (Fig. 3.3). The similarity of the mecA sequence among species of Proteus, Morganella, Enterococcus and Staphylococcus indicates that mecA is more widely distributed in the environment than previously understood and also hints at the potential for SCCmec transfer among differing bacteria genera. The transfer of SCCmec among genera is thought to be relatively rare. However, segments of PBP-encoding genes can be acquired by marginally-resistant bacteria including Haemophilus, Neisseria and Streptococcus (Dowson et al., 1994), effectively increasing their resistance to β-lactam antibiotics and the significance of environmental reservoirs of pathogens with increased resistance to antibiotics (Dowson et al., 1994). Of further importance is the possibility that infected or colonized people from the community (e.g. bathers) who seek medical attention might carry these pathogens to the healthcare setting, facilitating their spread and survival in
clinical niches that are characterized by high antibiotic pressure (Chambers, 2001; Carleton et al., 2004). This same mechanism of spreading pathogens from the community to the clinic is thought to have driven the increased clinical occurrence and success of community-acquired methicillin-resistant *S. aureus* (Chambers, 2001).

![Sequence Alignment Diagram](image)

**Figure 3.3**: An example of the alignment of the sequence of *mecA* from the nonstaphylococcal isolates (dotted arrow) with *mecA* from MRSA (above dotted arrow).

BOX-PCR analysis generates distinctive DNA fingerprints by amplifying segments of repetitive genetic elements (BOX) that are randomly dispersed throughout an organism’s genome. Since the repetitive elements in differing strains of a given bacteria species can be uniquely positioned in the genome, BOX-PCR fingerprinting can
differentiate bacteria species and/or strains (Versalovic et al., 1991; Rademaker et al., 1998). In this study, BOX-PCR genotyping showed that the \textit{mecA}-containing isolates of \textit{P. vulgaris} and \textit{E. faecalis} were genetically diverse within each species and did not belong to a single dominant strain. Specifically, the \textit{P. vulgaris}, \textit{M. morganii} and \textit{E. faecalis} isolates exhibited six, one and two unique BOX-PCR fingerprints, respectively (Fig. 3.4), suggesting that the pathogens originated in different pollution sources (Dombek et al., 2000) and might collectively exhibit phenotypic traits that result in higher virulence and therefore increased ability to impact the health of a host (Abu-Raddad et al., 2008).
Figure 3.4: BOX-PCR of DNA isolated from bacteria containing meca. Legend: Marker, 100 bp DNA ladder; lanes 1-6, the six genotypes representing the P. vulgaris isolates; lane 7, the one genotype representing the M. morganii isolates; lane 8-9, the two genotypes representing the E. faecalis isolates.

P. vulgaris, M. morganii and E. faecalis are known to exhibit resistance to multiple antibiotics commonly prescribed to treat symptoms similar to the infections caused by these pathogens (CLSI, 2006). Antibiotic sensitivity assays revealed that all of the isolates were resistant to three β-lactam antibiotics including oxacillin (MIC > 128 mg l\(^{-1}\)), ampicillin (MIC > 128 mg l\(^{-1}\)), and amoxicillin (MIC > 128 mg l\(^{-1}\)). All isolates were also resistant to antibiotics representing other classes including glycopeptides (vancomycin (MIC > 128 mg l\(^{-1}\))), macrolides (erythromycin (MIC > 128 mg l\(^{-1}\))), and tetracyclines (tetracycline (MIC > 32 mg l\(^{-1}\))). Although tolerance to glycopeptides and macrolides is expected for the Enterobacteriaceae, the resistance patterns observed here suggested that multiple resistance mechanisms characterize these environmental pathogens. All of the isolates were susceptible to trimethoprim (MIC < 8 mg l\(^{-1}\)) and ciprofloxacin (MIC < 1 mg l\(^{-1}\)). The antibiotic susceptibility of the control strains was also assayed to determine if the environmental isolates exhibited unique susceptibility patterns. For all three species, the environmental strains exhibited resistance to a greater number of antibiotics than the control strains. Specifically, P. vulgaris (ATCC 6896) was susceptible to tetracycline, M. morganii (NRRL B-1663) was susceptible to erythromycin and tetracycline, and E. faecalis (ATCC 29212) was moderately resistant to oxacillin and tetracycline, but susceptible to all other antibiotics tested. These results are of particular interest, as they not only illustrate a difference in resistance between control and
environmental strains of the same bacteria, but also show that \textit{mecA} can be maintained in natural reservoirs.

Despite efforts to isolate methicillin-resistant staphylococci, none were detected in these natural surface waters. However, DGGE analysis was performed to determine the complexity of the bacteria community that was resistant to oxicillin (methicillin). DGGE generated multiple bands from the DNA of each sample, showing that each water site harbored an assembly of several oxacillin-resistant bacteria. The diagnostic bands that represented \textit{P. vulgaris} and \textit{E. faecalis}, respectively, were present in almost all the samples (n=11), while bands diagnostic for \textit{M. morganii} were present in fewer samples (n=6), further confirming the occurrence of these pathogens as part of the resistant community (Fig. 3.5). Comparison of the community DGGE fingerprint to those generated from pure isolates of \textit{S. aureus}, \textit{P. vulgaris}, \textit{M. morganii} and \textit{E. faecalis} showed that \textit{S. aureus} were absent from any of the profiles representing the enrichments (Fig. 3.5), confirming the absence of \textit{S. aureus} (and MRSA) from the enriched water samples. Spurious matching of bands can occur in DGGE leading to false identification of the occurrence of the pathogens in water samples. However, the DGGE analysis was used in this study to confirm the data obtained from the multiplex-PCR analysis, and mainly to provide evidence for the absence of \textit{S. aureus}. While DGGE analysis is an effective method for detecting substantial changes in diverse microbial assemblages (Muyzer \textit{et al.}, 1993), it should be noted that many bacteria species harbor multiple copies of the 16S rRNA gene, which results in multiple bands representing a single species in the DGGE fingerprints (Dahllöf \textit{et al.}, 2000). Consequently, this might lead to
overestimating the complexity of the bacterial community occurring in the samples (Dahllöf et al., 2000).

Figure 3.5: DGGE analysis of methicillin-resistant bacteria communities. DGGE analysis of a known isolate of *S. aureus* generated a band that did not co-migrate with any of the bands from the sites. The DGGE marker was prepared from DNA of pure type strains available in the laboratory. Sites correspond to the locations from where the water samples were collected (see map above).

The absence of methicillin-resistant staphylococci could be attributed to deficiencies in the selection media (Stepan, 2004), limited bacteria fitness resulting from the physiological burden of antibiotic-resistance in these pathogens (Chambers, 2001) or
prevailing environmental conditions. For example, ultraviolet radiation represents a crucial factor affecting the survival of MRSA, as exposure efficiently killed 99.9% of MRSA inoculum after as low as 5 seconds of exposure and 100% at 90 and 120 seconds (Conner-Kerr et al., 1998).

The role of mecA in the pathogens isolated in this study is not clear and might not be related to antibiotic resistance. Wu et al. (1996) showed that the DNA isolated from over 150 independent isolates of S. sciuri hybridized with a mecA probe. DNA analysis revealed sequences that were 88% similar to mecA harbored by methicillin-resistant -S. aureus and -S. epidermidis (Wu et al., 1996). However, most of the S. sciuri isolates harboring the mecA homologue were susceptible to methicillin and penicillin, suggesting that the mecA homologue might encode a function in S. sciuri that was not related to resistance to methicillin (Wu et al., 1996). However, the detection of mecA in nonstaphylococcal pathogen might shed new light on the distribution and occurrence of this gene, which were previously confined to staphylococci.

In conclusion, these results underscore the role of the natural environment as a reservoir of clinically-significant pathogens that harbor mecA. The pathogens isolated in this study highlight the public health significance of bacteria pollution in the natural environment (Rózalski et al., 1997). Significantly, the waters used in this study were historically associated with higher level of indicators of fecal pollution and implicated in outbreaks of disease (Dorfman and Stoner, 2007). Bacteria were reported as the major etiological agents in fecally-polluted waters and were responsible for 62 waterborne diseases (48.4% of which were gastroenteritis and 11.3% were acute respiratory illnesses), causing illnesses in 2,698 individuals (Dorfman and Stoner, 2007). Combined
with the historic implication of these waters in disease outbreaks, these results further emphasize the need for environmental pathogen surveillance to support ongoing epidemiology programs.
Chapter 4

Detection and differentiation of mixed Staphylococcus bacteria using denaturing gradient gel electrophoresis (DGGE)

Abstract

Although a few Staphylococcus spp are components of the normal human flora, under conditions of compromised immunity, several staphylococci can cause serious infections. Therefore, detecting and identifying species of staphylococci from the environment of at-risk patients is an important step in limiting infections. We developed a novel denaturing gradient gel electrophoresis (DGGE) protocol for the identification of staphylococci that takes advantage of Staphylococcus spp.-specific polymorphisms in the gene that encodes elongation factor Tu (tuf). PCR analysis of tuf was performed on DNA isolated from pure cultures of 27 different staphylococci. DGGE analysis effectively separated the PCR products into 19 different band positions, including unique positions for important species such as S. aureus, S. hominis, S. lugdunensis, S. warneri, S. capitis, S. caprae and S. saprophyticus. DGGE also differentiated Staphylococcus spp. in artificially-mixed assemblages compromised of 3 randomly selected Staphylococcus species. Application of the protocol was demonstrated by swabbing clinical surfaces (n=16) in patient’s rooms and clinical surfaces (n=16) that were subjected to
decontamination. DGGE analysis of the *tuf*-PCR product performed on the DNA isolated from the clinical surfaces showed that the surfaces were contaminated with several species of staphylococci, including *S. aureus*, *S. epidermidis*, *S. lugdunensis*, *S. hominis* and *S. simulans* and others. The identity of the staphylococci was further confirmed by commercial sequencing of the DGGE bands. A similar DGGE analysis of clinical surfaces before and after decontamination showed that several staphylococci persisted in spite of the decontamination efforts. DGGE of *tuf* represents a promising technique to detect, characterize and monitor mixed assemblages of staphylococci to facilitate epidemiological studies and decontamination efforts.

**Introduction**

The staphylococci are gram-positive bacteria that often occur as a part of the harmless bacterial community inhabiting the skin and nasal cavities of humans and animals (Kloos and Bannerman, 1994; Huebner and Goldmann, 1999; Hanssen and Ericson Sollid, 2006; Martins and Cunha, 2007). Of the *ca.* 31 species of staphylococci currently recognized, 15 are potentially pathogenic to humans (Kloos and Bannerman, 1994; Martins and Cunha, 2007) and these are often implicated in healthcare- and community-acquired illnesses, ranging from mild skin infections to life threatening diseases and death. Since staphylococci are capable of acquiring a remarkable range of resistance to antibiotics, staphylococcal infections can be recalcitrant to antibiotic chemotherapy. Antibiotic-resistant staphylococci have become a world-wide problem that not only impacts public health, but also affects the health care system itself by causing prolonged hospitalization and increases in costs of treatments and patient mortality (Cosgrove *et al.*, 2003 and 2005; Engemann *et al.*, 2003).
Historically, *S. aureus* has been regarded as the most important staphylococcal species in terms of public health (Sakai et al., 2004). For example, *S. aureus* and antibiotic-resistant *S. aureus* strains such as methicillin-resistant *S. aureus* (MRSA) are regarded as important causative agents of healthcare- and community-associated infections (Lowy, 1998; Zetola *et al*., 2005), resulting in skin and soft-tissue infections in addition to more serious respiratory (pneumonia), circulatory (bacteremia), central nervous system (meningitis), and other diseases (Lowy, 1998; Klevens *et al*., 2007). However, many other *Staphylococcus* species that were previously disregarded as insignificant clinical contaminants have gained increased attention as important human pathogens. For example, *S. epidermidis*, the most frequently isolated *Staphylococcus* species from human samples (Martins and Cunha, 2007), can cause a wide range of health problems, including infections of the bloodstream (bacteremia), throat, nose, ear, eye, cardiovascular system (e.g. prosthetic-valve endocarditis and intravascular catheter infections), surgical wounds, central nervous system, and infections associated with dialysis (Vuong and Otto, 2002; Heikens *et al*., 2005 and references therein). Other important non-*aureus* staphylococci include *S. saprophyticus*, the second most-frequently-isolated species in acute urinary-tract infections (Gupta *et al*., 1999; McTaggart *et al*., 1990), and *S. haemolyticus*, *S. hominis* and *S. lugdunensis*, which often contaminate blood samples and are associated with a variety of human infections (Froggatt *et al*., 1989; Grosserode and Wenzel, 1991; Kloos and Bannerman, 1994). The coagulase-negative staphylococci (CNS) are a subgroup of the staphylococci that include many clinically-important species that are sometimes collectively considered equally or more clinically-important than *S. aureus* (Huebner and Goldmann, 1999). For example,
approximately 38% of the isolates from bloodstream infections of intensive-care-unit patients were identified as CNS, while 13% were *S. aureus* (National Nosocomial Infections Surveillance (NNIS), 1999). Considering the broad pathogenic impact that the different *Staphylococcus* species have on human health, reliable surveillance and identification of staphylococci represents a vital step for infection control. This is especially important as staphylococcal species have been reported to occur in the hospital environment, contaminating surfaces and spreading infections to patients. For example, staphylococci have been isolated from hospital surfaces, including indwelling medical devices, computers, bed rails, countertops, floors, door handles, faucets, bed linens, tables, blood pressure cuffs/tourniquets, and gowns and gloves of healthcare personnel (Boyce *et al.*, 1997; Blythe *et al.*, 1998; Bures *et al.*, 2000; von Eiff *et al.*, 2002; Devine *et al.*, 2001; Neely *et al.*, 2005; Wilson *et al.*, 2006; Snyder *et al.*, 2008).

The detection and characterization of staphylococci contaminating clinical surfaces are important to assess the risk of infection and cleanliness in the patient’s environment. The identification of staphylococci to the species level can be achieved using biochemical and molecular methods. Popular biochemical methods include the API identification systems (Biomerieux) (Anderson *et al.*, 1983, Kloos and Wolfhohl, 1982), the BD Phoenix Automated Microbiology System, and the MicroScan automated system (Dade Behring Inc., Deerfield, Ill.) (Hussain *et al.*, 1986). These methods generally rely on a series of biochemical tests to identify staphylococci and are usually used singly or in combination with other techniques for the identification of clinical isolates (Anderson *et al.*, 1983; Kloos and Wolfhohl, 1982; Hussain *et al.*, 1986). However, in many cases these methods fail to provide clear identification and
differentiation between coagulase-negative species (Kloos and Bannerman, 1994 and 1995; Kloos and Wolfhohl, 1982). A further disadvantage of these systems includes the considerable time expenditure caused by the steps required for bacteria isolation, purification and growth (Martineau et al., 2001; Stepan, 2004). Numerous examples of molecular methods to identify *Staphylococcus* spp exist that predominantly focus on individual species such as *S. aureus*, *S. epidermidis*, and *S. saprophyticus* (e.g. Brakstad et al., 1993; Gaszewska-Mastalarz et al., 1997; Martineau et al., 1996 and 1998; Saruta et al., 1995; Zakrzewska-Czerwinska et al., 1992). Alternatively, some molecular methods (e.g. multiplex PCR analysis) can be used to identify multiple *Staphylococcus* species. For example, Morot-Bizot et al. (2004) used multiplex PCR for the simultaneous identification of *S. aureus*, *S. epidermidis*, *S. saprophyticus*, and *S. xylosus*. However, disadvantages to molecular methods (including multiplex PCR assays) also exist. For example, PCR-based identification relies on prior knowledge of genetic sequences in the targeted species in order to design species-specific PCR primers that allow the differentiation of multiple species in mixed samples (e.g. Morot-Bizot et al., 2004; Stepanovic et al., 2008). Several examples of genes exhibiting specificity for *Staphylococcus* species-exist (Goh et al., 1996 and 1997; Stepan, 2004). One gene target of particular interest is *tuf*, which encodes the elongation factor Tu and is involved in the formation of the peptide chain during protein synthesis (Grunberg-Manago, 1996). Targeting *tuf*, Martineau et al. (1998, 2001) developed a *Staphylococcus* species-specific PCR assay that could detect (but not differentiate) 27 staphylococcal species. In this system, differentiation of the staphylococci, including *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, and *S. saprophyticus*, was reliant on species specific *tuf-*
hybridization probes, a step that adds considerable complexity to the identification scheme. The utility of \textit{tuf} DNA sequences to distinguish the staphylococci to the species level derives from its species-specific sequence heterogeneity. As a result, \textit{tuf} gene sequencing was reported as an effective method to identify CNS and \textit{S. aureus} to the species level when compared to biochemical methods and sequencing of other gene targets (e.g. 16S rRNA gene) (Heikens \textit{et al.}, 2005; Martineau \textit{et al.}, 2001).

Healthcare-associated contamination (i.e. hospital surfaces and indwelling devices) with bacteria often involves multiple staphylococci (Boyce \textit{et al.}, 1997; von Eiff \textit{et al.}, 2002; Devine \textit{et al.}, 2001; Neely \textit{et al.}, 2005; Wilson \textit{et al.}, 2006; Snyder \textit{et al.}, 2008). However, identifying these organisms using existing methodologies (e.g. hybridization probe or sequencing assays) requires considerable time and expense. This is especially of concern in an outbreak situation in which many samples need to be collected and processed for rapid identification of staphylococci. To address this limitation, a molecular fingerprinting system was developed that uses denaturing gradient gel electrophoresis (DGGE) analysis that effectively separates \textit{tuf}-specific PCR products generated from a mixed assemblage of staphylococci. This DGGE-analysis generates a molecular fingerprint in which the unique sequence-based position of the bands in the gel would correspond to different staphylococcal species, allowing the characterization of multiple staphylococci in a single assay without prior colony isolation. Therefore, in this study, we (i) investigated the effectiveness of DGGE of \textit{tuf}-PCR to detect and differentiate 27 staphylococcal species, (ii) demonstrated the use of this method in context of characterizing \textit{Staphylococcus} species contaminating clinical surfaces, and (iii) evaluated decontamination efforts that targeted these clinical surfaces.
Materials and Methods

Staphylococcal species and bacterial cultures:

Pure cultures of twenty-seven different species of staphylococci were obtained from the American Type Culture Collection (ATCC) and the US Agricultural Research Service Culture Collection (Northern Regional Research Laboratory (NRRL), Peoria, IL) (Table 4.1). All samples were resuscitated from lyophilized preparations by inoculation in Muller-Hinton (MH) broth and incubation at 35° C for 24-36 h with shaking. Glycerol stocks of each isolate were prepared and stored at -80° C for future use. Fig. 4.1 describes the overall flow of experiments conducted in this study.

![Diagram of experimental flow](https://via.placeholder.com/150)

Figure 4.1: A schematic showing the flow of the experiments conducted in this study.
Table 4.1: List of *Staphylococcus* species used in this study and the natural hosts that commonly harbor them (Kloos and Bannerman, 1994). NRRL: Northern Regional Research Laboratory.

ATCC: American Type Culture Collection.

<table>
<thead>
<tr>
<th>Staphylococcal Species (n=27)</th>
<th>Strain Identifier</th>
<th>Natural Host</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus arlettae</em></td>
<td>NRRL B-14764</td>
<td>Mammals, birds</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> subsp. <em>aureus</em></td>
<td>ATCC 6538</td>
<td>Humans, mammals, birds</td>
</tr>
<tr>
<td><em>Staphylococcus auricularis</em></td>
<td>ATCC 33753</td>
<td>Humans</td>
</tr>
<tr>
<td><em>Staphylococcus capitis</em> subsp. <em>capitis</em></td>
<td>NRRL B-14752</td>
<td>Humans, some primates</td>
</tr>
<tr>
<td><em>Staphylococcus caprae</em></td>
<td>NRRL B-14757</td>
<td>Humans, goats</td>
</tr>
<tr>
<td><em>Staphylococcus carnosus</em> subsp. <em>carnosus</em></td>
<td>NRRL B-14760</td>
<td>Meat and fish products, unknown</td>
</tr>
<tr>
<td><em>Staphylococcus chromogenes</em></td>
<td>NRRL B-14759</td>
<td>Cattle, horses, goats</td>
</tr>
<tr>
<td><em>Staphylococcus colnii</em> subsp. <em>colnii</em></td>
<td>NRRL B-14756</td>
<td>Humans, primates</td>
</tr>
<tr>
<td><em>Staphylococcus delphini</em></td>
<td>NRRL B-14767</td>
<td>Dolphins</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>ATCC 146</td>
<td>Humans (domestic mammals)</td>
</tr>
<tr>
<td><em>Staphylococcus equorum</em></td>
<td>NRRL B-14765</td>
<td>Horses, cattle</td>
</tr>
<tr>
<td><em>Staphylococcus felis</em></td>
<td>NRRL B-14779</td>
<td>Cats</td>
</tr>
<tr>
<td><em>Staphylococcus gallinarum</em></td>
<td>NRRL B-14763</td>
<td>Poultry, birds</td>
</tr>
<tr>
<td><em>Staphylococcus haemolyticus</em></td>
<td>NRRL B-14755</td>
<td>Humans, primates, domestic mammals</td>
</tr>
<tr>
<td><em>Staphylococcus hominis</em> subsp. <em>hominis</em></td>
<td>NRRL B-14737</td>
<td>Humans</td>
</tr>
<tr>
<td><em>Staphylococcus intermedius</em></td>
<td>ATCC 29663</td>
<td>Mammals, birds</td>
</tr>
<tr>
<td><em>Staphylococcus kloosii</em></td>
<td>NRRL B-14766</td>
<td>Mammals</td>
</tr>
<tr>
<td><em>Staphylococcus lentus</em></td>
<td>ATCC 49574</td>
<td>Domestic mammals, dolphins</td>
</tr>
<tr>
<td><em>Staphylococcus lugdunensis</em></td>
<td>NRRL B-14774</td>
<td>Humans</td>
</tr>
<tr>
<td><em>Staphylococcus pasteuri</em></td>
<td>NRRL B-23827</td>
<td>Humans, goats</td>
</tr>
<tr>
<td><em>Staphylococcus saccharolyticus</em></td>
<td>NRRL B-14778</td>
<td>Humans</td>
</tr>
<tr>
<td><em>Staphylococcus saprophyticus</em></td>
<td>ATCC 49907</td>
<td>Humans, mammals</td>
</tr>
<tr>
<td><em>Staphylococcus schleiferi</em> subsp. <em>schleiferi</em></td>
<td>NRRL B-14775</td>
<td>Human infections, unknown, dogs</td>
</tr>
<tr>
<td><em>Staphylococcus sciuri</em> subsp. <em>sciuri</em></td>
<td>NRRL B-14777</td>
<td>Mammals, birds</td>
</tr>
<tr>
<td><em>Staphylococcus simulans</em></td>
<td>NRRL B-14753</td>
<td>Humans, mammals</td>
</tr>
<tr>
<td><em>Staphylococcus warneri</em></td>
<td>ATCC 49454</td>
<td>Humans, primates, domestic mammals</td>
</tr>
<tr>
<td><em>Staphylococcus xylosus</em></td>
<td>ATCC 35663</td>
<td>Humans, mammals, birds</td>
</tr>
</tbody>
</table>

**DNA isolation and quantification:**

DNA was isolated from the cultures of each isolate. Briefly, 2 ml of each culture was centrifuged in a 2 ml microcentrifuge tube at 13,000 x g for 2 min, following which the supernatant was discarded. The cell pellet was resuspended in 1 ml of sterile DNA extraction buffer (pH 8) (50 mM NaCl, 50 mM Tris-HCl (pH 7.6), 50 mM EDTA, and 5% sodium dodecyl sulfate (SDS)) and 0.5 ml of 0.1 mm glass beads (Biospec Products Inc., OK, USA). The tubes were shaken in a bead beater (Fastprep, Bio101,
Thermosavant, MN, USA) for 30 s at 5.5 ms⁻¹ (Sigler and Zeyer, 2004). DNA was purified using the phenol (pH 8)/chloroform extraction method as described in Sambrook and David (2001). The isolated DNA was quantified by measuring optical density at A₂₆₀ and diluted with DNA-grade water to an average concentration of 100 μg ml⁻¹ and stored at -20° C before further use.

**tuf-PCR analysis:**

PCR analysis of *tuf* was performed on each *Staphylococcus* DNA sample according to the method of Martineau *et al.* (1998, 2001) using primers F-5’-GGCCGTGTTGAACGTGGTCAAATCA-3’ and R-5’-TIACCATTCAGTACCTTCTGGTAA-3’. Briefly, 1 μl of DNA template, 1× PCR buffer supplemented with 1.5 mmol L⁻¹ of MgCl₂ (Fisher Scientific, PA, USA), 1.5 mg ml⁻¹ bovine serum albumin (BSA), 0.2 mmol L⁻¹ of each dNTP, 0.02 U μl⁻¹ of Taq polymerase, and DNA-grade water were combined to achieve a final volume of 50 μl. The thermal-cycling conditions were 1 cycle at 95° C (5 min), followed by 30 or 40 cycles at 95° C (1 min), 55° C (1 min), and 72° C (1 min), and final extension at 72° C (30 min). The optimal number of cycles was determined empirically to enhance weak PCR products. All sets of reactions included a positive control sample containing *Staphylococcus aureus* DNA and a negative control sample containing no DNA template. PCR products (5 μl) were screened by electrophoresis in a 1% agarose gel containing 0.5 μg ml⁻¹ ethidium bromide. To determine if the correct PCR product (370 bp) was obtained, the size of the products from all the samples including the positive control was determined by comparison to a 100 bp DNA-ladder marker. To facilitate the best
separation of PCR products during DGGE analysis, a GC-clamp (Muyzer et al., 1993) was attached to either the forward or the reverse primer, respectively. The position of the GC-clamp on the primer (either the forward or reverse fragment) was chosen based on position that resulted in the best separation of the *Staphylococcus* isolates. The sensitivity and specificity of the GC-clamped primers was compared to that of the non-GC primers to determine the effect of the clamp on the PCR reaction. The specificity of the PCR primers was tested by performing PCR analysis (as described above) on DNA isolated from each bacterium in a collection of non-staphylococci. The test isolates were selected as a random subset from the larger collection that was tested by Martineau et al. (2001) and included *Acinetobacter baumannii, Agrobacterium tumefaciens, Bacillus subtilis, Enterococcus casseliflavus, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Morganella morganii, Ralstonia picketti, Pseudomonas aeruginosa, Proetus mirabilis, Proteus vulgaris, Salmonella enteritidis, Salmonella enterica, Salmonella typhi, Shigella dysenteriae, and Shigella flexneri*. The PCR performed on the DNA from the nonstaphylococcal species resulted in no visible product when screened in a 1% agarose gel with the exception of *Enterococcus casseliflavus*, which showed a PCR product identical in size (370 bp) to that generated from the DNA of the staphylococcal species.

**Sensitivity of the *tuf*-PCR assay:**

The sensitivity of the PCR assay was tested by performing the analysis on serial dilutions of whole cells and DNA from each of the 27 *Staphylococcus* species. Briefly, each *Staphylococcus* species was cultured for 16 h with shaking (180 rpm) in MH broth,
the culture was then centrifuged at 13,000 x g and the resulting pellet was washed twice with sterile, 10 mM sodium phosphate buffer (pH 7.0). The cells were serially diluted (10-fold), achieving a range from uncountable number of cells to 1-10 cells per µl of buffer, representing the lowest number of cells achieved by dilution. The number of cells was determined by plating 100 µl from each dilution onto MH agar plates and incubating for 24-48 h 35º C. DNA was isolated from each dilution and subjected to tuf-PCR analysis as described above.

In a separate analysis, the sensitivity of the tuf-PCR was also determined by using the DNA that was previously isolated from each species. The DNA was serially diluted (10-fold), achieving a lowest concentration of 0.01 ng of DNA per PCR reaction.

**DGGE analysis of tuf-PCR products:**

PCR analysis targeting the tuf gene was performed on the DNA isolated from each of the 27 *Staphylococcus* spp, followed by DGGE analysis according to the method of Muyzer *et al.* (1993). All DGGE analyses were performed with a DCode Universal Mutation Detection System (Bio-Rad, USA) in gels containing a denaturant gradient of 30–42% (100% denaturant represents 7 M urea and 40% vol/vol formamide), which was determined empirically and allowed optimal separation of the pure *Staphylococcus* species. Analysis of the position of each band generated from the individual *Staphylococcus* spp was carried out using GelCompar II software (Version 3.5, Applied Maths), with a band position tolerance of 0% and optimization of 0.5% (Esseili *et al.*, 2008).
To determine the sensitivity of the *tuf*-DGGE method in low-bacterial-abundance scenarios, DGGE analysis was performed on artificially-mixed DNA of three *Staphylococcus* species (*S. aureus*, *S. warneri*, and *S. saccharolyticus*). These 3 species were selected because they were clinically important and generated bands that were visually well-separated on the DGGE. For this purpose, equal volumes of DNA from the 3 different *Staphylococcus* species were mixed and serially diluted (10-fold) in sterile DNA-grade water. Two µl from each dilution was then analyzed with PCR and DGGE as described above. To determine if PCR/DGGE detection is biased toward abundant DNA, artificial assemblages were also prepared by mixing the DNA of *Staphylococcus* species in different ratios. For example, DNA from 3 different *Staphylococcus* species was mixed in to achieve the following ratios (by volume): 1:1:1; 2:1:1; 2:2:1; 3:1:1; 3:2:1; 3:3:1; 3:2:2; 5:1:1; 5:5:2; 10:1:1; 10:5:1; 10:5:2 and 2 µl from each mix was analyzed with PCR and DGGE as described above.

**tuf-DGGE analysis of DNA isolated from clinical surfaces contaminated with staphylococci:**

To test the utility of the *tuf*-DGGE method, 16 clinical surfaces were sampled (Table 4.2 and 4.3) during operation hours from an isolation room that housed a patient diagnosed with a MRSA infection at the University of Toledo Medical Center. Additionally another 16 clinical surfaces from a similar room were sampled before and after daily cleaning procedures using conventional mop-and-bucket methods. Samples were collected with transport swabs (Fisher Scientific, Inc., Pittsburgh, PA) and were stored at approximately 4° C before analysis (~2 h). To facilitate comparison between
samples collected before and after decontamination, each surface was divided into two equal areas, each of which sampled either before or after cleaning. The samples were enriched with shaking (180 rpm) in MH broth and incubated at 35°C for 18 hrs. DNA was isolated from each of the samples that exhibited bacterial growth and subjected to tuf-DGGE analysis as described above.

To confirm the identities of the species that were detected by tuf-DGGE, fifty-eight DNA bands (n = 58) in the resulting DGGE fingerprints were excised and incubated in sterile water at 4°C for 24 h to elute the DNA. PCR analysis of the DNA targeting tuf was performed as described above using non-GC clamped primers. The PCR product was purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) and sequenced in both directions (MWG Biotech Inc. (NC, USA)). The putative identity of the organism most closely associated with each sequence was determined using the BLAST algorithm (Altschul et al., 1990). DNA sequences were deposited in the National Center for Biotechnology Information Genbank database (Genbank accession numbers: in process).

**Results**

**1. tuf primer specificity and sensitivity:**

The specificity of the tuf primer was tested on a collection of bacteria, including staphylococcal and non-staphylococcal species, and revealed that under the described PCR analysis conditions, amplification was successful for each of the DNA templates isolated from each *Staphylococcus* species. However, PCR analysis of 17 non-
staphylococcal species resulted in the positive amplification of the DNA from one species, *Enterococcus casseli­flavus* (data not shown).

The sensitivity of the *tuf* PCR analysis was determined using serially-diluted DNA concentrations from each of the 27 *Staphylococcus* species used in this study. For all species, the PCR reaction produced the appropriate size product (370 bp) when an average of 0.001 ng or more of DNA was used per 50 µl of PCR reactions (final DNA concentration was equivalent to 20 fg µl⁻¹). The *tuf* PCR sensitivity was also tested by performing PCR analysis on serial dilutions of whole cells of each *Staphylococcus* species. In all cases, a *tuf*-PCR product of the appropriate size was observed when PCR reactions contained an average of 1 or more cells. The GC-clamped *tuf* primers exhibited similar sensitivity in the PCR analysis as compared to that of the non-GC primers (data not shown).

**2. DGGE analysis and discrimination of Staphylococcus species:**

DGGE analysis of the DNA from each *Staphylococcus* isolate showed that 13 staphylococci, including clinically-important species such as *S. aureus*, *S. hominis*, *S. lugdunensis*, *S. warneri*, *S. capitis*, *S. caprae*, and *S. saprophyticus*, maintained unique band-positions in the DGGE gel (Fig. 4.2). However, the *tuf*-PCR products from each of the remaining 14 species were resolved to 6 unique band positions in the DGGE. Specifically, the product of i- *S. arlettae* co-migrated with that of *S. saccharolyticus*, ii- *S. epidermidis* co-migrated with *S. kloosii*, iii- *S. schleiferi* co-migrated with *S. haemolyticus*, iv- *S. felis* co-migrated with *S. cohnii*, *S. delphini*, and *S. intermidus*, v- *S.
chromogenes co-migrated with *S. simulans*, and vi- *S. auricularis* co-migrated with *S. xylosus*.

Figure 4.2: DGGE analysis of *tuf*-PCR products generated from DNA of 27 pure and pre-identified *Staphylococcus* species. Boxes encompass the bands representing the species that co-migrated and...
did not maintain a unique band position in the DGGE gel. The staphylococci marker includes all the combined \textit{tuf}-PCR products from all 27 staphylococcal species. The 30\%-40\% denaturing gradient was used to achieve optimal separation of the bands.

3. **DGGE analysis of mixed assemblages of \textit{Staphylococcus} species:**

   DGGE analysis of mixed staphylococci was tested by combining the PCR products (1:1:1 ratio) of three different staphylococci and performing DGGE analysis on the artificially mixed DNA. In most cases, DGGE analysis differentiated \textit{tuf} into distinct bands that represented the different species in the artificial assemblage (data not shown). In cases when the artificial assemblage contained the products of species that co-migrated, a single band was observed that represented these species. DGGE analysis of the mixed \textit{tuf}-PCR product from all pure isolates generated a profile that consisted of 19 separate bands. This profile was subsequently used as a marker (Fig. 4.3) for all further comparative analyses of mixed staphylococci assemblages (artificial and natural).
Figure 4.3: DGGE profile representing 27 pre-identified *Staphylococcus* species that were separated into 19 unique band positions. Bands that are labeled with the more than one species name represent the band positions of the species that co-migrated in the DGGE. The 30%-40% denaturing gradient was used to achieve optimal separation of the bands. This marker was used for characterization and of staphylococci from unknown samples.

To determine the sensitivity of the DGGE method in low-bacterial-abundance scenarios, equal masses of DNA from three *Staphylococcus* species (e.g. *S. aureus*, *S.
warneri, and S. saccharolyticus) was mixed, serially diluted (10 fold to 1:1000,000) and subjected to PCR and DGGE analyses as described above. Results showed that DGGE analysis detected and differentiated three bands from all dilutions with the exception of the 10⁻⁶ dilution (equivalent to 0.00003 ng µl⁻¹ of DNA), at which the bands were no longer visible (Fig. 4.4).

Figure 4.4: DGGE analysis of tuf-PCR product generated from three Staphylococcus species (S. aureus, S. warneri, and S. saccharolyticus). DNA from these species was artificially mixed and serially
diluted (10-fold) prior to performing *tuf*-DGGE analysis. $10^{1}$ dilution contained 30 ng µl$^{-1}$ of DNA prior to PCR, while the $10^{6}$ contained 0.00003 ng µl$^{-1}$ of DNA. The marker is the staphylococci marker that was made from all the staphylococci used in this study and described in Fig. 4.2. The DGGE was made of 30%-40% denaturing gradient.

To determine if preferential amplification of proportionally abundant DNA occurs in this system, the DNA from three *Staphylococcus* species known to exhibit clearly separated bands following DGGE analysis (e.g. *S. aureus*, *S. warneri*, and *S. carmpsis*) was artificially mixed in different ratios and subjected to PCR and DGGE analyses. In all cases, DGGE analysis separated the PCR products into the expected three bands, representing the three species in the mixture (Fig. 4.5).
Figure 4.5: DGGE analysis of *tuf-PCR* product generated from three *Staphylococcus* species (*S. aureus*, *S. warneri*, and *S. carmipsis*). DNA from these species was artificially mixed (volume to volume) to achieve different ratios prior to *tuf*-DGGE analysis. The marker is the staphylococci marker that was made from all the staphylococci used in this study and described in Fig. 4.2. The DGGE was made of 30%-40% denaturing gradient.
4. Application of the method: DGGE analysis of staphylococci on clinical surfaces:

tuf-DGGE analysis was performed to characterize potential staphylococci contaminating clinical surfaces. DGGE analysis of the tuf-PCR product showed that at least one Staphylococcus spp occurred on all 15 of the sampled surfaces, while seven surfaces were each contaminated with at least two different species of staphylococci (Fig. 4.6). The major species detected on the surfaces using both DGGE and sequencing were S. epidermidis, S. simulans, S. lugdunensis, and S. aureus (Table 4.2). Specifically, S. epidermidis, S. lugdunensis, S. simulans, and S. aureus occurred on 8, 5, 3 and 2 surfaces, respectively. The most highly-contaminated surfaces in terms of number of species detected were the bathroom rail and dry-erase eraser, which each harbored three species of staphylococci, including S. epidermidis, S. lugdunensis, and S. aureus.
Figure 4.6: DGGE profiles of tuf-PCR products generated from clinical surfaces samples. Numbered bands were further identified following excision, DNA isolation and sequencing (Table 4.2). The marker is the staphylococci marker that was made from all the staphylococci used in this study and described in Fig. 4.2. The DGGE was made of 30%-40% denaturing gradient.

Table 4.2: List of Staphylococcus species that were detected on clinical surfaces. These species were identified using DGGE and identity was confirmed by sequencing of DGGE bands. The band number refers to the label assigned to bands that were excised from the DGGE fingerprints (see Fig. 4.6).
Further application of the tuf-DGGE analysis was used to characterize the effectiveness of cleaning processes that are used as daily procedures within a medical center. For this purpose, another 16 surfaces in a patient’s room were sampled before and after clean-up. Results showed that the cleaning efforts did not eliminate all contaminating staphylococci (Fig. 4.7). Specifically, all samples (including those collected before and after cleaning) harbored at least one *Staphylococcus* species (Fig. 4.7). Three surfaces (faucet-2, patient light switch, and toilet seat) harbored a contaminating staphylococci assemblage, including *S. epidermidis* and *S. lugdunensis*, that was identical on all three surfaces before and after decontamination (Fig. 4.7). In

<table>
<thead>
<tr>
<th>Surface Name</th>
<th>Band Number</th>
<th>Closest Identity</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linens</td>
<td>1</td>
<td><em>Staphylococcus simulans</em></td>
<td>99</td>
</tr>
<tr>
<td>Chair Handle</td>
<td>2</td>
<td><em>Staphylococcus simulans</em></td>
<td>100</td>
</tr>
<tr>
<td>Bed Rails</td>
<td>3</td>
<td><em>Staphylococcus simulans</em></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>93</td>
</tr>
<tr>
<td>Pillow</td>
<td>5</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>99</td>
</tr>
<tr>
<td>I.V. Box Buttons</td>
<td>6</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td>Faucet</td>
<td>7</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>99</td>
</tr>
<tr>
<td>Blood Pressure Cuff</td>
<td>12</td>
<td><em>Staphylococcus lugdunensis</em></td>
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</tr>
<tr>
<td>Shelves</td>
<td>8</td>
<td><em>Staphylococcus epidermidis</em></td>
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<td></td>
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<td></td>
<td>14</td>
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</tr>
<tr>
<td>Bathroom Rail</td>
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<td><em>Staphylococcus lugdunensis</em></td>
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</tr>
<tr>
<td></td>
<td>16</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>93</td>
</tr>
<tr>
<td>Dry-Erase Eraser</td>
<td>17</td>
<td><em>Staphylococcus aureus</em></td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td><em>Staphylococcus aureus</em></td>
<td>94</td>
</tr>
</tbody>
</table>

89
several cases (cabinet handles, floor, sink countertop, faucet-1, linens), the samples collected after cleaning harbored species that were not present prior to cleaning. The staphylococcal species that were detected in samples collected prior to and after cleaning of the same surface included \textit{S. epidermidis} (occurred on toilet seat, patient switch light, and door handle), \textit{S. haemolyticus} (on toilet seat), and \textit{S. lugdunensis} (on main light switch, patient light switch, toilet seat, and faucet-2) (Table 4.3). \textit{tuf}-DGGE and sequencing of DNA in the DGGE bands showed that the before and after decontamination samples harbored a variety of clinically-important staphylococcal spp, including \textit{S. epidermidis} (occurred in 17 of the 32 samples), \textit{S. lugdunensis} (15 samples), \textit{S. hominis} (2 samples), \textit{S. simulans} (1 sample), \textit{S. auricularis} (1 sample), \textit{S. haemolyticus} (3 samples), and \textit{S. aureus} (1 sample).
Figure 4.7: DGGE analysis of *tuf*-PCR products generated from clinical surfaces samples collected before (A) and after (B) daily room cleaning. Numbered bands were further identified following excision, DNA isolation and sequencing (Table 4.3). The marker is the staphylococci marker that was made from all the staphylococci used in this study and described in Fig. 4.2. The DGGE was made of 30%-40% denaturing gradient.

Table 4.3: List of *Staphylococcus* species that were detected on clinical surfaces before (A) and after decontamination (B) efforts. These species were identified using DGGE and identity was confirmed by sequencing of DGGE bands. Band numbers refer to the bands that have been cut from the DGGE fingerprints (see Fig. 4.7).
<table>
<thead>
<tr>
<th>Surface Name</th>
<th>Pre (A)/Post (B)- decontamination</th>
<th>Band Number</th>
<th>Closest Identity</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.V. Box Buttons</td>
<td>B</td>
<td>1</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>2</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td>Bed Rails</td>
<td>B</td>
<td>3</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td>Blood Pressure Cuff</td>
<td>A</td>
<td>4</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5</td>
<td><em>Staphylococcus simulans</em></td>
<td>100</td>
</tr>
<tr>
<td>Toilet Cover</td>
<td>A</td>
<td>6</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7</td>
<td><em>Staphylococcus aureus</em></td>
<td>100</td>
</tr>
<tr>
<td>Table</td>
<td>A</td>
<td>8</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>9</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
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<tr>
<td>Main light Switch</td>
<td>A</td>
<td>10</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>94</td>
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<tr>
<td></td>
<td>B</td>
<td>11</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td>Linens</td>
<td>A</td>
<td>13</td>
<td><em>Staphylococcus hominis</em></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>14</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>99</td>
</tr>
<tr>
<td>Toilet Seat</td>
<td>A</td>
<td>16</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td><em>Staphylococcus haemolyticus</em></td>
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</tr>
<tr>
<td></td>
<td>B</td>
<td>19</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>94</td>
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<tr>
<td></td>
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<td>20</td>
<td><em>Staphylococcus auricularis</em></td>
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<td></td>
<td>21</td>
<td><em>Staphylococcus epidermidis</em></td>
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<td></td>
<td></td>
<td>22</td>
<td><em>Staphylococcus haemolyticus</em></td>
<td>96</td>
</tr>
<tr>
<td>Patient Light Switch</td>
<td>A</td>
<td>23</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>95</td>
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<tr>
<td></td>
<td></td>
<td>24</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>25</td>
<td><em>Staphylococcus lugdunensis</em></td>
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<tr>
<td></td>
<td></td>
<td>26</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td>Faucet-1</td>
<td>A</td>
<td>27</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>28</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29</td>
<td><em>Staphylococcus epidermidis</em></td>
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<tr>
<td>Sink Countertop</td>
<td>A</td>
<td>30</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>31</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td>Faucet-2</td>
<td>A</td>
<td>32</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>33</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td>Door Handle</td>
<td>A</td>
<td>34</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>35</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td>Floor</td>
<td>A</td>
<td>36</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>B</td>
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<td>96</td>
</tr>
<tr>
<td>Cabinet handles</td>
<td>A</td>
<td>38</td>
<td><em>Staphylococcus haemolyticus</em></td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>39</td>
<td><em>Staphylococcus hominis</em></td>
<td>98</td>
</tr>
<tr>
<td>Purell Dispenser Handle</td>
<td>B</td>
<td>40</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>97</td>
</tr>
</tbody>
</table>

**Discussion**
Analysis of *tuf* can provide sensitive and specific detection of staphylococci (Martineau *et al*., 2001; Stepan, 2004; Heikens *et al*., 2005), while species-specific sequence heterogeneity of *tuf* can be used to identify staphylococci to the species level (Martineau *et al*., 2001). The goal of this study was to expand on these methodologies to develop a method that could allow for the detection and monitoring of multiple species of staphylococci in a single assay. This was accomplished by adapting denaturing gradient gel electrophoresis (DGGE) to separate staphylococci species-specific *tuf* segments to characterize mixed assemblages of staphylococci.

**Ability of the *tuf*-DGGE assay to characterize single and mixed species of staphylococci:**

As expected from exploiting the species-specific *tuf* sequence heterogeneity, DGGE analysis of the *tuf*-PCR product showed that DGGE could efficiently detect and differentiate the *Staphylococcus* species, most of which exhibited unique band positions (Fig. 4.2). Of particular importance, the analysis successfully differentiated the most clinically-important species, including *S. aureus*, *S. hominis*, *S. lugdunensis*, *S. warneri*, *S. capitis*, *S. caprae*, and *S. saprophyticus* (Fig. 4.2). Some clinically-important and infection-causing *Staphylococcus* species such as *S. epidermidis*, *S. cohnii*, *S. saccharolyticus*, and *S. simulans* were not easily differentiated, as their representative DGGE bands were found to co-migrate with species that were not frequently associated with a human host (Fig. 4.2). Specifically, the band representing *S. epidermidis* co-migrated with that of *S. kloosii*, a bacterium normally isolated from mammals (Kloos and Bannerman, 1994). While the band representing *S. cohnii* co-migrated with that of *S.
delphini, S. felis, S. intermidus, which are commonly isolated from dolphins, cats and mammals and birds, respectively (Kloos and Bannerman, 1994). Additionally, the band representing S. saccharolyticus co-migrated with that of S. arlettae, which is isolated from mammals and birds, while the band representing S. simulans co-migrated with S. chromogenes, which is isolated from cattle, horses and goats (Kloos and Bannerman, 1994). Also, the band representing S. haemolyticus co-migrated with that of S. schleiferi, while that of S. auricularis co-migrated with S. xylosus, and all of these species are associated with humans (Kloos and Bannerman, 1994), although S. xylosus is also commonly associated with mammals and birds and has been occasionally identified as a cause of human infection (Kloos and Bannerman, 1994). Since staphylococci that were not associated with humans and/or infection are not expected to be frequently isolated from clinical samples, it follows that the clinically important isolates that co-migrated with the non-human associated species can still be uniquely identified in the DGGE gel. This assumption was further confirmed by sequencing the DGGE bands generated from clinical surfaces (Table 4.2 and 4.3), which showed that all the bands belonged to species associated with humans (Kloos and Bannerman, 1994; Martins and Cunha, 2007). Consequently, the comparison of DGGE profiles of staphylococci from unknown clinical samples to the profiles or band positions of the pre-identified species (combined into a marker, Fig. 4.3) would in most cases facilitate the detection and characterization of clinically important staphylococci that might occur on contaminated clinical surfaces.

The sensitivity of the tuf-DGGE analysis was exhibited in efficiently detecting and characterizing multiple species of staphylococci that were artificially mixed (Fig. 4.4 and 4.5). Furthermore, the tuf-DGGE analysis successfully characterized samples that
contained lower DNA concentrations from the mixed staphylococcal assemblages, which further confirmed the suitability of this assay even in cases when samples might contain relatively lower numbers of staphylococcal cells. Since staphylococci are important nosocomial pathogens that can survive in the hospital environment (Marshall et al., 1998; Neely and Maley, 2000; Martins and Cunha, 2007), detecting health risk-associated staphylococci is important in the clinical settings to maintain a risk-free environment and reduce the transmission of these pathogens to patients (Scott and Bloomfield, 1990; Guyot and Layer, 2006; Boyce, 2007). Consequently, \textit{tuf}-DGGE assay might provide a sensitive monitoring tool for assessing environmental contamination with staphylococci and determining the frequency and suitability of decontamination to achieve a safe environment for patients.

**Ability of the tuf-DGGE to characterize staphylococci occurring on clinical surfaces:**

Since \textit{Staphylococcus} species often occur on clinical surfaces and pose an infectious risk, \textit{tuf}-DGGE analysis was used to determine the number of species harbored on common clinical surfaces in a hospital room. DGGE analysis and sequencing of DNA from the DGGE fingerprints of staphylococci communities found on clinical surfaces (Fig. 4.6) showed that \textit{S. epidermidis}, \textit{S. simulans}, and \textit{S. lugdunensis} were the prominent contaminants (Fig. 4.6). The identified species are all opportunistic pathogens capable of causing serious infections, especially in immuno-compromised individuals (Kloos and Bannerman, 1994; Huebner and Goldmann, 1999; Martins and Cunha, 2007 and references therein), a group that makes up a large proportion of hospital patients. \textit{S. epidermidis} and \textit{S. lugdunensis} were the most frequently identified \textit{Staphylococcus} spp.
This is important, as coagulase-negative staphylococci (CNS) such as *S. epidermidis*, *S. simulans*, and *S. lugdunensis* are known to cause a wide range of health problems, including bloodstream (bacteremia), throat, nose, ear, eye, cardiovascular (e.g. prosthetic-valve endocarditis and intravascular catheter infections), surgical wound, central nervous system, dialysis-associated, and prosthetic-joints infections (Vuong and Otto, 2002; Heikens *et al.*, 2005 and references therein). The detection of these species on clinical surfaces was not surprising, as *S. epidermidis* and *S. lugdunensis* are among the most-frequently-isolated CNS from humans (Heikens *et al.*, 2005). For example, Huebner and Goldmann (1999) reported that 65% to 90% of the staphylococci occurring in human samples were identified as *S. epidermidis*, which can explain the prevelance of this bacterium on the surfaces sampled in this study.

**Using *tuf*-DGGE to assess the efficiency of decontamination of clinical surfaces:**

To determine the suitability of the *tuf*-DGGE method to assess the efficiency of decontamination used in a clinical setting, samples were collected from surfaces in an occupied isolation room before and after daily mop-and-bucket cleaning procedures. DGGE analysis showed that many clinically-important *Staphylococcus* species occurred after decontamination (Fig. 4.7), suggesting that an enhanced approach might be needed to efficiently decontaminate the surfaces. These results support the findings of others, who have reported that conventional cleaning does not always remove pathogens from contaminated surfaces and that improved methods for cleaning are needed (Boyce, 2007). Since surfaces were divided into two areas, one of each sampled before and after cleaning, the presence in post-cleaning samples of *Staphylococcus* species that were not
present in pre-cleaning samples could be attributed to heterogeneity in the distribution of bacteria on the surfaces.

Many approaches have been suggested for proper eradication of staphylococcal species. For example, general-purpose detergent (Allen et al., 1997), vacuuming and damp dusting (Masterton et al., 1995), hydrogen peroxide vapour (French et al., 2004), and gaseous ozone (de Boer et al., 2006) were among the approaches suggested for eradication of methicillin-resistant \textit{S. aureus}. While the efficacy of decontamination strategies was not the focus of this research, the choice of cleaning can impact patient health and the hospital budgets (Boyce, 2007; de Boer et al., 2006). For this reason, it is possible that the DGGE developed in this study can provide an effective means to monitor contamination/decontamination, enabling healthcare providers to adopt efficient strategies that provide safer healthcare.

**The comparison of \textit{tuf}-DGGE analysis to other methods:**

As a molecular method, \textit{tuf}-DGGE analysis avoids traditional (phenotype) isolate-based identification methodologies that have been shown to be inadequate for the identification of most CNS (Heikens et al., 2005). In addition, the \textit{tuf}-DGGE assay provides a relatively complete survey of the species that might occur in a given sample, as compared to biochemical approaches that often suffer from isolation-media biases throughout. Furthermore, some staphylococci, due to defects in metabolism, can reduce their growth rate and exhibit a small-colony phenotype, which often cannot be detected by conventional microbiological techniques (Krimmer et al., 1999 and references therein). DGGE has been successfully used to characterize complex bacteria assemblages,
avoiding the need to collecting and screening large libraries of isolates. For example, DGGE analysis of *Escherichia coli* communities was used to bypass the collection of extensive *E. coli* isolate libraries (Esseili *et al.*, 2008). Additionally, *tuf*-DGGE analysis can detect and characterize more species as compared to the single-species PCR analysis, multiplex-PCR analysis (Morot-Bizot *et al.*, 2004), and species-specific nucleotide-probe-based methods (Martineau *et al.*, 200), all of which are also excessively labor intensive (Stepan, 2004). DNA-based molecular techniques that are not culture dependent can detect the DNA of dead staphylococcal cells, consequently leading to overestimation of the risk of bacterial contamination (Masago *et al.*, 2008). To ensure that the *tuf*-DGGE analysis did not detect the DNA that belonged to non-viable or dead cells (e.g. Masago *et al.*, 2008), the clinical samples were cultures before by *tuf*-DGGE analysis; hence the species that were detected on the surfaces by *tuf*-DGGE were viable and capable of growth, and capable of causing disease. The *tuf*-DGGE method can allow the identification of some *Staphylococcus* spp (e.g. *S. aureus*, *S. hominis*, *S. lugdunensis*, *S. warneri*, *S. capitis*, *S. caprae*, and *S. saprophyticus*) (Fig. 4.2), bypassing the need for gene sequencing, which is dependent on gene banks that might not contain optimal sequences (Heikens *et al.*, 2005).

In terms of efficiency, approximately, 38 hours are required following sampling to generate a DGGE fingerprint that characterizes the staphylococci community. Specifically for 1 sample, 16 h are required for enrichment, 3 h for DNA isolation and a *tuf*-PCR assay, and then 18 h for setting up and running DGGE gels. In comparison, conventional and biochemical techniques, such as the API test strips, usually require between 24 and 48 h isolating a growing colony and an additional 24 h of incubation,
resulting at least 48-72 h for standard identification of an isolate. However, further confirmatory analyses might require additional time (Renneberg et al., 1995; Rhoden and Miller, 1995). Although DGGE has been amply used as a fingerprinting tool, it should be noted that DGGE does require some training for inexperienced personnel. Consistency in gel preparation and electrophoresis is crucial to generate useful DGGE fingerprints (Osborn and Smith, 2005). However, using customized markers (in this case the *Staphylococcus* marker) (Fig. 4.3) and computer software (e.g. Gelcompar software that was used in this study) to standardize gels enhances the analysis.

**Specificity and sensitivity of the tuf-PCR primers:**

The *tuf*-PCR assay has been successfully used in the detection of a variety of species, including *Mycoplasma fermentans* (Berg et al., 1996), *M. pneumoniae* (Luneberg et al., 1993), and enterococci (Ke et al., 1999). The *tuf* gene PCR primers that were developed by Martineau et al. (2001) to detect staphylococci were selected for this study because: i- they exhibit high specificity and sensitivity (Martineau et al., 2001), and ii- the size of *tuf*-PCR product was 370 bp (Martineau et al., 2001), which is in the recommended size range for use in DGGE analysis (Osborn and Smith, 2005). Additionally, in this study it was shown that DGGE analysis of *tuf*-PCR product generated from DNA of pure staphylococcal culture yielded a single diagnostic band for each species, and that DGGE of *tuf*-PCR products generated from DNA of pure staphylococcal culture resulted in optimal separation of the staphylococcal species to uniquely-positioned bands in the gels, which further emphasized the importance of the *tuf*-gene as a target for this study. Other potential targets included PCR primers for the
sodA gene (Heikens et al., 2005) and a larger fragment of the tuf gene (412 bp) (Heikens et al., 2005), both of which were previously sequenced resulting in successful identification of Staphylococcus species (Heikens et al., 2005; Poyart et al., 2001). These PCR primers were tested but not selected for this study, as DGGE analysis of the sodA gene generated multiple bands for each staphylococcal species, preventing appropriate fingerprinting. The DGGE analysis of the larger tuf fragment did not result in optimal separation of the PCR-product from pure staphylococci into unique positions in the gels (data not shown).

The tuf gene primers selected for this study were necessarily supplemented with a GC-clamp to facilitate DGGE analysis (Muyzer et al., 1993). However, the GC-clamp did not affect the sensitivity and specificity of the primers. Martineau et al. (2001) reported a weak cross-amplification with the DNA from E. faecalis and Macroccocus caseolyticus (55 non-staphylococcal species were tested), but our analysis showed no cross-amplification with these species. However, cross-amplification with Enterococcus casseliflavus, which was not tested by Martineau et al. (2001), was noted. Enterococcus casseliflavus is part of the normal gastrointestinal flora and can cause disease in patients (Iaria et al., 2005). Furthermore, this Enterococcus species has been reported to occur and survive in a clinical environment (Neely and Maley, 2000). However, DGGE analysis showed that the tuf-PCR product generated using DNA from Enterococcus casseliflavus did not align with any Staphylococcus species present in the DGGE marker. Our GC-clamped primers exhibited a detection limit comparable to that reported in Martineau et al. (2001), which estimated a detection limit of 5 to 10 S. aureus genome copies when using a 40-cycle PCR reaction. Attaching the GC-clamp to the forward tuf
primer resulted in relatively better DGGE separation of the pure staphylococci *tuf*-PCR product as compared to the attaching the GC-clamp to the reverse primer (data not shown).

**Conclusions:**

*tuf*- DGGE analysis is a promising method to characterize mixed assemblages of staphylococci. It improves on existing methodologies by avoiding (i) the isolation of staphylococci on agar plates that are primarily designed for the identification of a single species (e.g. *S. aureus*), (ii) biochemical tests that have limited use for identifying CNS, and (iii) labor-intensive hybridization probes and gene sequencing. The results of this study also showed that *tuf*- DGGE analysis can improve the monitoring of important management practices in the healthcare environment. For example, DGGE analysis can enable healthcare staff to employ the most efficient management methods (e.g. decontamination of clinical surfaces), to provide their patients with a more healthy environment.
Chapter 5
Conclusions and Future Research

Many *Staphylococcus* species can occupy different niches in/on the human body, mostly as commensals. However, under conditions of compromised immunity, many staphylococcus species can cause serious infections (Kloos and Bannerman, 1994; Huebner and Goldmann, 1999; Hanssen and Ericson Sollid, 2006; Martins and Cunha, 2007). Historically, staphylococci are considered among the leading causes of hospital-associated infections, while more recently a new trend in staphylococcal (*e.g.* *S. aureus*) infections that affects the non-clinical community has emerged (Lowy, 1998; Zetola *et al.*, 2005; Chambers, 2001, Kluytmans-VandenBergh and Kluytmans, 2006). The colonization of the human body (*e.g.* skin) with staphylococci facilitates the dispersal of these species to other human hosts and their environment through shedding and direct contact. Consequently, environmental contamination (*i.e.* high contact shared surfaces such as computer keyboards and clinical surfaces, and secondary environments that are polluted with human wastes such as recreational waters) with staphylococci poses a risk of serious infections for susceptible individuals such as the immuno-compromised, the elderly and/or new-borns (Kloos and Bannerman, 1994; Huebner and Goldmann, 1999; Foster, 2004). For this dissertation, three projects were designed to detect and identify staphylococci from three different environmental matrices (public computer...
keyboards, recreational waters, and clinical surfaces) in an effort to characterize reservoirs for these pathogens that have either been overlooked or not thoroughly studied. The identification and characterization of staphylococcal reservoirs other than the human hosts would be important to understand the changing epidemiology of these pathogens and the routes of their transmission in the human environment.

In the first study, multiplex-PCR techniques were developed to detect and identify staphylococci, including methicillin-resistant staphylococci, on computer keyboard surfaces used by students and staff in a metropolitan university. The results of this study showed that computer keyboards were reservoirs for clinically-important staphylococci, including methicillin-resistant *S. aureus* (MRSA), methicillin-resistant *S. epidermidis* (MRSE) and methicillin-resistant *S. hominis* (MRSH). The extensive use of computers by a relatively large population of people and the absence of public awareness of good hygiene practices (washing hands, cleaning keyboards) might facilitate the spread of these pathogens in the community. Additionally, this might shed light on the role of computer keyboards as reservoirs that might facilitate increased infection risk, as these and similar tactile surfaces are ubiquitous not only in the community, but also in the healthcare facilities. Although the number of community staphylococcal infections is increasing, little effort has been made to identify community reservoirs (Stepanovic *et al.*, 2008) of these pathogens, especially on high-contact inanimate surfaces. Historically, similar studies that investigated the role of inanimate surfaces as reservoirs for staphylococcal pathogens have been largely confined to the clinical environment and primarily focused on methicillin-resistant *S. aureus* (MRSA). Consequently, this study
identified an important reservoir and provided further evidence to warrant studies to identify contaminated surfaces in the community.

In the second study, fresh recreational waters (Bay Village, OH) that were impacted by fecal pollution and consequently important in spreading infections in swimmers were investigated for the presence of staphylococci. Natural waters receive human-related pollution, chemical and biological, either from surface run-off, sewage treatments, or recreation activities. Although swimmers have been reported to exhibit staphylococcal-like infections after bathing in contaminated waters (Gatermann and Crossley, 1997), and few studies reported the isolation of important staphylococcal species from sea water (Papadakis et al., 1997; Gabutti et al., 2004; Diaper and Edwards, 1994; Porter et al., 1993), the role of fresh water as a potential reservoir was not previously investigated. Although antibiotic-resistant staphylococci were not isolated from the fresh water that was collected from a recreational beach (Huntington Beach, OH), a PCR signal diagnostic of staphylococci was detected, indicating that the isolation media that were used to culture staphylococci might have been not adequate for isolation of these pathogens (Stepan, 2004). However, other antibiotic-resistant bacteria were isolated, including \textit{P. vulgaris}, \textit{M. morganii}, and \textit{E. Faecalis}, all of which are known human pathogens. These pathogens were multi-antibiotic resistant and carried \textit{mecA}, a gene conferring resistance to a wide range of \(\beta\)-lactam antibiotics, which was previously associated only with staphylococci. The results of this study showed that \textit{mecA} is more widespread in the environment than previously thought (previous studies associated a \textit{mecA} homologue to naturally occurring non-\textit{aureus} staphylococci). The isolation of bacteria that were resistant to multiple clinically-important antibiotics, including
vancomycin and methicillin, and carried the \textit{meca} from a secondary environment was of particular significance, as these species (especially \textit{E. faecalis}) are more commonly associated with a clinical environment.

In the third study, a community fingerprinting method (DGGE) was customized to allow the specific and sensitive detection and characterization of assemblages of \textit{Staphylococcus} species that contaminated surfaces in the clinical environment. The detection and characterization of staphylococci is crucial for diagnosis of infection, determining the severity of clinical contamination, and assessing the efficiency of available decontamination methods in maintaining an infection risk-free environment. Although about 15 species of staphylococci are associated with human infection (Kloos and Bannerman, 1994; Huebner and Goldmann, 1999), available methodologies for their characterization are inadequate, limited to the detection of few species, highly technical, expensive, and/or time consuming (Stepan, 2004; Heikens \textit{et al}., 2005). DGGE analysis showed that most surfaces in an isolation room housing a patient were contaminated with single or multiple species of clinically important staphylococci, including \textit{S. aureus}, \textit{S. epidermidis}, \textit{S. simulans}, \textit{S. hominis}, and \textit{S. lugdunesis}. DGGE analysis was not only successful in detecting the contaminating species, but also highlighted the prevalence and identity of these species in the clinical environment. Furthermore, DGGE analysis showed that daily room cleaning procedures were not sufficient to eradicate all staphylococci. This is an important finding, as now healthcare staff have a tool with which they can assess prevalence of contamination in their facilities and the efficiency of contamination management, which would allow for a healthier patient environment.
Finally, this thesis represented studies concerned with aspects of public health microbiology and attempts to develop methodologies to identify pathogen reservoirs in environmental matrices and achieve a better understanding of contamination with staphylococci. The methods and findings reported in this thesis can be used to further expand future studies of contamination with staphylococci. For example, the tuf-DGGE method can be used to extensively sample admitted patients in order to identify carriers of important staphylococci and quarantine the patients before they contaminate the hospital environment and spread the bacteria to others. Alternatively, discharged patients could be also tested to limit the spread of the pathogens to the community. The methodologies employed in this thesis can also facilitate decontamination efforts of important clinical surfaces and devices, assess the efficiency of drugs used for decolonization of staphylococci from the nares, or monitor the response of staphylococci inhabiting the skin to cleaning products and antiseptic creams in hosts, including immuno-compromised patients. In addition, other potential community reservoirs such as domesticated animals, the farm environment, polluted natural environments or human waste, and numerous high contact surfaces (ATM machines, door handles, public transportation system and many others) can be tested for staphylococcal contamination to understand the spread of these bacteria and minimize the risk of infection. Finally, the possibility for lateral gene transfer of mecA between staphylococcal and non-staphylococcal pathogens should be determined, as mecA-containing non-staphylococcal pathogens might act as reservoirs, promoting the rise of antibiotic resistant staphylococci in hospitals and communities.
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