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Abstract

Joseph Everette Hatch: Methicillin-resistant *Staphylococcus aureus* in Gyms: Evaluation of Swabbing-Based Sampling Methodologies and the Levels of MSSA/MRSA on Environmental Surfaces within Recreational Exercise Facilities

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is an emerging infectious bacterial pathogen which is a leader in the causes of nosocomial infections. Over time, this pathogen has transitioned from association primarily with healthcare settings to association with community-acquired infections (CA-MRSA) as well. As CA-MRSA prevalence has risen, its outbreaks have been linked to common environmental sources; prompting interest in the environment as a potential means of its dissemination. To investigate this risk in gyms, four types of sampling devices were evaluated. The findings were used to create an environmental sampling protocol, with the goal of estimating the prevalence of MRSA. Sampling was by swabbing a surface area with a moistened gauze wipe, enrichment culture of recovered wipe fluid, platting of enrichment cultures on differential/selective media and colony isolation and identification. Of 8 facilities sampled, 5 had MRSA (1.69-10% prevalence) and the overall MRSA prevalence for all samples was 4.5%.

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III. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an emerging infectious bacterial agent that first developed with the introduction of semi-synthetic penicillins (e.g. methacillin, oxacillin) and has continued to acquire new resistances to a wider and wider array of antibiotics as they are developed. *Staphylococcus aureus* and MRSA both cause a wide variety of diseases ranging from simple types of dermatitis to systemic infections, food poisoning, respiratory infection, and toxic-shock syndromes. Originally only associated with the healthcare system, MRSA also emerged in the community and now causes a large proportion of community-acquired infections in addition to being one of the main causes of healthcare associated infections in the United States.

Recent outbreaks of MRSA in the healthcare and community settings have sparked an interest in the possibility that the environment may play some role in the transmission of these pathogens between individuals. Before outbreaks linked to environmental transmission, it was thought that person-to-person transmission was the only route of spread as this is a human pathogen that has no known environmental o animal reservoirs. Studies performed in the healthcare environment have demonstrated that MRSA can be found in the environments of patient wards as well as other locations, while investigations of community based environments have largely yielded negative findings.

Many reported outbreaks of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) have occurred in participants of sports teams, prompting an interest in the possibility that shared equipment was acting as a means of

transmission in these incidents. These types of reports often prompt stories in the media which sensationalizes these accounts and incites public concern about the possibility of MRSA inside public recreational exercise facilities.

This study was aimed at investigating the prevalence and levels of MRSA contamination on exercise equipment inside gyms in Orange, Durham, and Wake counties of North Carolina in an effort to address these concerns. Before we could undertake field sampling, candidate sampling methods were first evaluated in order to understand their relative efficacies and learn more about the potential problems with interpretation of the results obtained during environmental sampling. Much is unknown about the true distribution and fate of these organisms on environmental surfaces, but much of the work already done in the context of food safety and bioterrorism investigation industries can be used to inform this and future investigations.

IV. Research Objectives

- Evaluate the current status and need for improved environmental sampling protocols in order to incorporate methods that would allow for enhanced and predictable recovery and detection of MSSA/MRSA
- Evaluate and compare the efficacies and other performance characteristics of potential sampling devices in the recovery of MSSA/MRSA from environmental surfaces
- Obtain field data on the prevalence of MSSA/MRSA contamination to estimate actual contamination levels on environmental surfaces within recreational exercise facilities
- Obtain and interpret information about which types of facilities or equipment may have higher rates of MRSA
- Draw conclusions about factors influencing the prevalence and contamination levels of environmental surfaces with MRSA.

V. Literature Review

A. MRSA: the Organism

1. What is *Staphylococcus aureus*?

Bacteria of the genus *Staphylococcus* can be both commensals and pathogens of humans. Staphylococci are found mainly on the skin, outer mucous membranes (e.g. in the nares), and in the gastrointestinal tract of humans and animals. They also can be found contaminating the hands, forearm, axilla, pharynx, the skin of the trunk, and the perianum/groin/vagina (77). They are Gram-positive cocci which produce the enzyme catalase and are negative for oxidase production. They are largely benign and even thought to be beneficial to the human host in that they may help prevent colonization with some harmful microorganisms. However, there are a few species in the genus that are pathogenic to humans with *Staphylococcus aureus* the pathogenic species of greatest public health importance.

Staphylococcus aureus is found colonizing the nares of approximately 32% of the U.S. population (44) and is distinguished from other staphylococci by its production of coagulase. Colonization is asymptomatic, but when *S. aureus* is introduced into a site that provides a route for invasion, like a scrape or cut, or if it colonizes an immunocompromised host, it can cause a range of infections from dermatitis to systemic infection and septicemia. Some strains can elaborate toxins that can cause toxic shock syndrome or scalded skin syndrome.

S. aureus gets its name based upon the yellow-golden colorization that its colonies usually take on during incubation on nutrient-rich agar such as blood agars. Most *S. aureus* isolates are also beta-hemolytic on such agars, which aids in their clinical identification.

2. What is MRSA?

Penicillin was commercially released for civilian use in the mid-1940's, and the first strains of penicillin-resistant *Staphylococcus aureus* appeared as early as 1947. The next generation of penicillin-based drugs (semi-synthetic beta-lactam drugs) came into use quickly thereafter: methicillin and oxacillin. The first strains of methicillin-resistant *Staphylococcus aureus* were reported in 1961 in the United Kingdom.

Methicillin-resistant *Staphylococcus aureus* (MRSA), also commonly referred to as multi-drug resistant *Staphylococcus aureus* or oxacillin-resistant *Staphylococcus aureus* (ORSA) has become increasingly resistant to a wider and wider range of antibiotic options. Today, approximately 0.8% of the U.S. population is colonized with MRSA at any one time (44). Infections caused by MRSA include the same types commonly caused by MSSA (methicillin-susceptible *Staphylococcus aureus*) but are more recalcitrant to treatment, requiring more aggressive and expensive types of antibiotics and additional laboratory work-up. This leads to increased disease severity in many cases as well as increased medical costs. MRSA can lead to very serious diseases including necrotizing fasciitis or pyomyocitis, necrotizing pneumonia, and endocarditis, but like all *S. aureus* infections, is most commonly encountered as less severe (although still serious)

infections of the upper to mid-levels of the skin in the form of rashes and/or non-healing pus filled boils, which, if left untreated can develop into more serious disease.

3. Antibiotic Resistance

MRSA, and other antibiotic resistant organisms, emerged largely in response to the use and over use of antibiotics; which introduced selective pressure for the development and subsequent dissemination of antibiotic resistance factors among bacterial populations. Types of antibiotic resistance include: *Intrinsic Resistance*, where a microorganism has naturally occurring or consistently inherited factors which confer resistance to an antibiotic, and *Acquired Resistance*, where a microorganism develops a resistance either through a genetic mutation or by acquiring genetic material from other organisms through transformation, transduction, or bacterial conjugation in which chromosomal or plasmid elements can be transferred. Pathways for resistance can follow one or a combination of pathways: enzymatic degradation/modification of the antimicrobial; decreased uptake/accumulation of the antimicrobial; alteration of the antimicrobial agent's target; circumventing the antimicrobial agent's effects; or uncoupling of the antimicrobial agent and target interactions with downstream effects on metabolism. (27)

4. Antibiotic Resistance in MRSA

After the introduction of penicillin, it did not take long for *staphylococci* to develop resistance to this first generation antibiotic by acquisition of the *blaZ* gene encoding for the beta-lactamase enzyme (61) which inactivates penicillin. Later, when semi-synthetic penicillin-based beta-lactam drugs were introduced in 1959-1960 (methicillin and oxacillin), some *Staphylococcus aureus* strains developed resistance to these drugs when they underwent alterations in the target of all beta-lactam based antibiotics, their penicillin-binding proteins (PBPs). PBPs are transpeptidases that function in the crosslinking of peptidoglycan subunits in the bacterial cell wall. Beta-lactam-based antibiotics bind to these PBPs, blocking their ability to function, and leaves the bacteria unable to repair, grow, or divide.

Some strains of *S. aureus* were able to obtain the ability to produce a modified PBP known as PBP2a, which is a high-molecular weight, multi-domain protein which has a low affinity for all beta-lactam based drugs (48). PBP2a is encoded by the *mecA* gene which is part of the genetic element known as *SCCmec* or the staphylococcal cassette chromosome *mec* (61). *SCCmec* may have originated in a coagulase-negative staphylococcal species such as *Staphylococcus haemolyticus* or *Staphylococcus sciuri* (Hiramatsu *et al.* 2001) or from *Staphylococcus epidermitis* (81).

The *SCCmec* element contains both a *mec* gene complex and a *ccr* gene complex. There are at least five types of *SCCmec* elements based on which combination of *mec* and *ccr* complex types that compose the element (I, II, III, IV, or V). Generally, those strains of MRSA commonly associated with hospitals and healthcare (HA-MRSA) are types I,

II, or III. Strains associated with community acquisition (CA-MRSA), which are theorized to have developed independently from HA-MRSA strains, are types IV and V usually. CA-MRSA strains also typically have other factors used to distinguish them from HA-MRSA strains including types of virulence factors, antibiotic resistance patterns, theorized source of acquisition, and the MRSA associated risk factors that the individual had. Typically, *SCCmec* types I, IV, and V do not carry any other antibiotic resistance genes, while types II and III potentially carry a wide array of resistance genes. Other antimicrobials to which MRSA has demonstrated resistance include the aminoglycosides (amikacin, gentamicin, tobramycin); quinolones (ciprofloxacin, levofloxacin, ofloxain); oxazolidinones (linezolid); macrolides (azithromycin, erythromycin), and lincosamides (clindamycin) as well as combination drugs like trimethoprim-sulfamethoxazole and quinupristin-dalfopristin. These resistances are mediated through various gene acquisitions or modifications. (61)

The development of multi-drug resistant MRSA strains has led to major concerns. vancomycin has long been the antibiotic of last resort for treatment of MRSA. No resistance to vancomycin was observed for many years, and doctors tried to prevent it from developing by only using it when absolutely necessary for multi-drug resistant MRSAs. However, even this last option is coming under pressure as a number of MRSA strains have developed intermediate resistance to vancomycin (VISA) and at least six cases of vancomycin-resistant *Staphylococcus aureus* infections have been documented in the US (68).

5. Virulence Factors and Pathogenesis

Staphylococcus aureus can carry a wide assortment of virulence factors which help it to colonize, infect and elicit disease. All *S. aureus* isolates, whether MSSA or MRSA, can exhibit any or all of these virulence factors; however, there are general associations between strain types of MSSA, HA-MRSA, and CA-MRSA and which virulence factors are typically found in isolates of these strains. Additionally, expression of various virulence factors can be influenced by the growth phase and growth conditions experienced by the organism through various gene induction/suppression mechanisms. (32)

Surface Bound Proteins
• Protein A-binds IgG antibodies to prevent opsonization
• PNSG-an adhesion
• Fibronectin binding protein
• Collagen binding protein
• Bound Coagulase-binds and coverts fibrinogen to fibrin
Secreted Proteins
• α-toxin-membrane pore forming hemolysin
• β-toxin-hydrolysis of cell wall lipids
• γ-toxin-wide range cytolytic activity
• Panton-Valentine Leukocidin—kills white blood cells through membrane lysis
• Exfoliate toxins (ETA, ETB)—cause epidermis sloughing
• Staphylococcal enterotoxins—gastrointestinal effects
• Toxic shock syndrome toxin (TSST-1)—leakage of endothelial cells
• Free Coagulase—converts fibrinogen to fibrin
• Deoxyribonuclease—hydrolyzes DNA
• Hyaluronidase—hydrolyzes connective tissue
• Lipases—hydrolyzes lipids
• Staphylokinase-lyses fibrin

Table 1: Virulence Factors of *Staphylococcus aureus*, adapted from Reygaert, W 2009

Colonization

Typically in staphylococcal infections, it is a colonizing strain of the organism that is responsible for disease production (80). Colonization provides a reservoir for the organism such that infection can occur once a break in the host defenses occurs. Such breaks usually take the form of a breach in the skin caused by some kind of trauma like shaving, surgical procedures, cuts and abrasions; but can also occur via aspiration of the organism (especially during other respiratory co-infections) or simple immunosuppression of the host. It has been shown by Wertheim *et al.* 2005 that colonization leads to an increased risk of infection (77).

Colonization by a *S. aureus* strain is mediated by a number of host-organism factors. The host factors associated with *S. aureus* colonization are poorly understood at this time. In the example of nasal colonization, such factors include secreted antimicrobial substances such as immunoglobulins, lysozymes, etc. (38) and the particular anatomy of the nasal passages are thought to be significant contributors to exposure-colonization outcomes. Additionally, it has been shown that *S. aureus* typically colonizes the section of the nares that is lacking in cilia and basically free from the antimicrobial substances normally secreted in mucus. (19,18, 77)

Organism factors that influence colonization include many of the virulence factors, particularly the surface bound proteins, reviewed in Table 1. The surface bound proteins related to effacement to host tissues are referred to as "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs). These MSCRAMMs allow the bacterial cells to attach to various tissues by adhering to substances such as collagen and fibronectin. Various assortments of these MSCRAMMs

in different *S. aureus* strains may mean that certain strains of *S. aureus* are more predisposed to effacement to certain tissues and thus more likely to cause certain types of disease. (32, 57)

Disease Production

A number of virulence factors enable some *S. aureus* strains to persistently colonize or cause disease in various individuals. This is accomplished by evading or counteracting host defenses. Examples of these virulence factors include: the ability to form biofilms (24), epithelial/endothelia cell invasion (53), the formation of small-colony variants (SCV's) which can lead to persistent/recurrent infection (60), the production of an antiphagocytic microcapsule (55), surface-bound protein A which prevents opsinization (61), and the secretion of chemotaxis inhibitory proteins which act to prevent neutrophil migration to the colonization/infection site (28).

Additionally, *S. aureus* can also produce a number of enzymes such as lipases, elastases, and proteases that help it break down host tissues and spread to additional sites within the host. Some strains produce a number of toxins that cause additional disease states such as septic shock, toxic shock syndrome, scalded skin syndrome/bullous impetigo, or food poisoning (32).

6. Epidemiology of MRSA

MRSA prevalence is on the rise in the United States as well as worldwide. In a study by Klevens *et al.* 2006, data from the National Nosocomial Infections Surveillance

system indicated that the number of *Staphylococcus aureus* isolates that were MRSA rose from 35.9% in 1992 to 64.4% in 2003; an increase of about 3% per year. Additionally, it has been estimated that approximately 2.3 million people, 0.8% of the population, carry MRSA in the US (44). Data from National Hospital Discharge Survey from 1999 to 2000 gave estimates of a total of 291,542 annual hospitalizations with a *S. aureus* related diagnosis; 125,969 of these (43.2%) were MRSA related, with most diagnoses of MRSA occurring in persons ≥65 years of age (43).

MRSA infection is largely associated with the health care system. Contact with the healthcare system and the use of antibiotics have long been known as risk factors for MRSA acquisition. In addition to being a very problematic nosocomial pathogen, MRSA has also developed and evolved in the community setting within the past couple of decades, infecting people without the typical risk factors for MRSA acquisition (31, 35). These new strains of MRSA that have arisen in the community are collectively referred to as community-acquired or CA-MRSA (healthcare associated strains are called HA-MRSA). Risk factors that have been identified for CA-MRSA include recent hospitalization, outpatient visit, nursing home admission, or antibiotic exposure, chronic illness, injection drug use, and close contact with an individual with aforementioned risk factors according to a meta-analysis conducted by Salgado *et al*. 2003.

Risk group/factor
Children <2 years of age
Athletes
Injection drug users
Men who have sex with men
Military personnel
Living in a correctional facility, residential homes, or shelters
Veterinarians, pet owners, and pig farmers
Adults ≥65 years of age
African-Americans
Recent influenza-like illness and/or severe pneumonia
Concurrent skin/soft tissue infection
History of colonization or infection with CA-MRSA
Close contact (often household) with a MRSA colonized individual

Table 2: Epidemiological Risk Factors for CA-MRSA infection, adapted from Boucher, H.W. 2008

CA-MRSA cases are on the rise in the US and other parts of the world. Outbreaks have been identified in children, the prison population, sports team participants, military personnel, men who have sex with men, and others (1, 8-11, 13) and have been recorded in North America, Latin America, Western Europe, Saudi Arabia, India, Asia, Australia, and New Zealand (4). It is thought that CA-MRSA strains originated from dispersion of HA-MRSA strains into the community, this seems to be the case for early CA-MRSA outbreaks. However, due to the fact that CA-MRSA strains appear very distinct from their HA-MRSA counterparts in the areas of non-beta-lactam

drug antibiograms, genotypes, and genetic determinants of virulence factors, it is hypothesized that contemporary CA-MRSA strains largely derived from community methicillin-susceptible *S. aureus* strains which horizontally acquired a *SCCmec* (type IV) element. Many of these strains have a distinct virulence factor, PVL (Panton-Valentine leukocidin) which aids in white blood cell destruction and makes these strains more virulent. This virulence factor is not typically found in HA-MRSA strains.(14, 15, 22, 23, 62, 75)

Distinguishing CA-MRSA strain features v. HA-MRSA
Absence of Healthcare associated risk factors Susceptibility to most non-beta-lactam antibiotics Distinct genotypes from HA-MRSA strains Presence of *SCCmec* type IV (and recently V) Distinct virulence genes (PVL and enterotoxins)

Table 3: Distinguishing CA-MRSA from HA-MRSA, as reviewed by Charlebois, E.D. 2004

After several years of circulation in the community, CA-MRSA strains have made their way into healthcare settings. They have begun to cause nosocomial infections like HA-MRSA strains (30). This may have serious implications for how future MRSA cases are handled as virulence factors and antimicrobial resistance traits of CA- and HA-MRSA strains are intermingled within the healthcare setting.

B. MRSA in the Environment

1. The Role of Surfaces in Disease Spread

Many human pathogens spend some time in the external environment between hosts. Many of these pathogens are carried between hosts via air or water, while others are transported via vectors (such as arthropods) or contaminated food. The modes of

environmental spread of various diseases have been thoroughly investigated (e.g. enteric pathogen transport in water); however, there has been increasing interest in the roles that environmental surfaces play in the spread of many pathogens. Fomites have been implicated in disease spread before, especially in healthcare and institutional environments, for pathogens such as the influenza virus (as reviewed by (76)), norovirus (12, 82) and others.

Recently, there has been an interest in the role of the environment in the spread of *Staphylococcus aureus* and MRSA that challenges the paradigm of strict person-to-person transmission of this pathogen. The first recognition of this phenomenon and most of the existing literature is from healthcare environments where there is great concern over MSSA/MRSA as a nosocomial pathogen (5, 6, 37, 40, 67). More recently, there has been an interest in environmental surfaces as reservoirs for the spread of MRSA in the community (17, 39, 49, 56, 65, 66, 70).

2. Deposition and Survival of *Staphylococcus aureus* in the Environment

Humans are the major reservoir of *Staphylococcus aureus* and thus, the major source of environmental staphylococci (16). This organism can be spread via the nose, mouth, skin, or gastrointestinal tract. There is a potential for aerial dissemination of staphylococci from the respiratory tract in colonized/infected individuals, especially under states of vigorous activity or severe disease; however, contamination of the hand and subsequent transfer to the environment is the most prominent source of environmental contamination.

In order for the environment to serve as a potential reservoir for a pathogen, the pathogen must be able to persist in the environment for some period of time. Pathogen survival in the environment is influenced by a multitude of environmental-organism interactions. Such factors as matter-phase, temperature, humidity, pH, presence of antimicrobials, the physical make-up and metabolic state of the pathogen as well as many other potential factors can determine the length of time a pathogen will remain viable in the environment.

Physiochemical	Biological	Pathogen Characteristics
Humidity	Predation	Pathogen Class and Structure
Salinity	Crowding	Resistances
pH	Presence of Nutrients	Metabolic needs
UV Radiation	Presence of Antimicrobials	Aggregation
Temperature		Dormant State
Organic and Inorganic molecules		
Matter-phase (composition/texture)		

Table 4: Factors Affecting Environmental Survival of Pathogens

Given these factors, it has been demonstrated that *Staphylococcus aureus* is able to remain viable under relatively normal physiological conditions on a number of various kinds of fomites ranging from dust particles to various surfaces that one may find in the healthcare setting such as fabrics and plastics for at least a matter of days to several months (16, 36, 37, 42, 51, 71, 74). It was previously thought that MRSA survival should resemble that of MSSA; Cimolai *et al.* (2008) postulated that there is little reason to suspect methicillin-resistance status should greatly affect environmental fitness. However, there is some evidence that some MRSA isolates may persist longer in the environment than some MSSA isolates (74). This work was done with a very limited number of isolates, making it difficult to determine the implications for MRSA in

general. Previous studies conducted in laboratory settings have demonstrated that *S. aureus* can survive from anywhere from a few hours to several months (See Table 5). Comparison across previous studies seems to suggest that both surface type and sample inoculation affect survival times of *S. aureus* (42).

Fomite	Survival	Inoculation	Source
Plastic Patient Chart Laminated Table Top Cloth Curtain	11 days >12 days 9 days	Not Specified	(37)
100% cotton (clothing), 100% cotton terry (towels and wash cloths) 60% cotton–40% polyester blends (scrub suits, lab coats, and clothing) 100% polyester (privacy curtains and clothing) 100% polyethylene plastic (splash aprons).	4-21 days 2-14 days 1-3 days 1-40 days 40->51 days	10^4-10^5cfu	(51)
Copper Pennies w/ blood and/or pus	<4hours ≥2 weeks	10^5cfu	(71)
Glass Container w/ and w/o Hospital Dust	>6mo	10^9cfu	(74)
Melamine Counter Top Vinyl Floor Tile Glass	0 -≥60days Increased with Inocula' Amount	50cfu-10^7cfu	(79)
Various Dry Surfaces (Literature Review)	7days-7mo.	Not Described	(42)

Table 5: Survival of *S. aureus* on Fomites

3. Evidence for MRSA in the Environment

The rise of MRSA as one of the leading causes of nosocomial infections prompted the first environmental investigations of MRSA prevalence focused on environmental surfaces in the healthcare setting. A multitude of such investigations have been conducted. Many studies yielded negative findings, while others suggest potentially ubiquitous *Staphylococcus aureus* in the healthcare environment and the possibility of environmental reservoirs for MRSA. (5, 6, 16, 36, 40, 46, 47, 54, 56, 58, 63, 67) Burn units especially may be of great concern as rates of environmental MRSA have been demonstrated in one study to be quite high and thus may pose serious risk to patients in these wards (63).

Surface	MRSA Prevalence	MRSA Concentration	Source
Various Surfaces in Regular Patient Wards	1-27%	N/A	(5) Review
Various Surfaces in MRSA colonized/infected Patients' rooms	27%	Not Given	(6)
Various Surfaces in an Emergency Department	2.5%	Not Given	(40)
Computer Keyboards and Mice in Hospital Wards	1.1%	Not Given	(46)
Room Door Handles in Wards	8.7%	$1\text{-}6x10^3$ /surface	(54)
Public Areas	0%	--	(56)
drug trolley, medication trolley, shelves of monitors and/or bed head	41%	0-6cfu /surface	(58)
Elevated Surfaces and Floors of Burn Unit Patient Rooms and Nearby Areas	31% 40%	$\leq$2.7cfu/cm^2 $\leq$5.3cfu/cm^2	(63)
Surfaces inside rooms of MRSA colonized patients	53.6%	Not Given	(67)

Table 6: Prevalence of MRSA on Surfaces in the Healthcare environment

Stemming from these inquiries, additional research has been done in the related fields of veterinary and animal sciences, examining veterinary wards (34) and various pet

and livestock animals for MRSA (21, 26, 41, 73). Municipal sewage has also been examined (3) as well as coastal marine waters (69). These studies have demonstrated that *S. aureus* and MRSA are present, at least in low levels either constantly or transiently, in all of these environments.

With the emergence of CA-MRSA, concern over environmental reservoirs of MRSA outside the healthcare setting has grown. A number of preliminary studies have attempted to find MRSA on surfaces commonly encountered in the community such as telephone mouthpieces (83), public transportation (70), computer terminals (39), various surfaces found in a university setting (7), and surfaces inside domestic residences (65, 66).

Surface	Prevalence	Source
Various Surfaces at a Large Urban University **-phone mouthpieces** **-elevator buttons** **-photocopier buttons** **-computer buttons** **-student desks** **-vending machine buttons**	0%	(7)
Exercise Equipment in gyms on a military base	0%	(29)
Computer Keyboards in University Computer Labs	8.3%	(39)
Surfaces in Kitchens, Bathrooms, and other surfaces in family homes	26% of homes had at least one positive surface 1.34% of all surfaces tested	(65)
Hand Railings in Public Transportation Vehicles	0%	(70)
High School Telephones	0%	(83)

Table 7: Prevalence of MRSA on Surfaces outside the healthcare environment (MRSA concentrations were not provided in these studies)

Most of the results from community sources have yielded very low to undetectable results for the amount of environmental MRSA there may be in these

settings. There are still concerns about the potential for environmental reservoirs of MRSA as there is some evidence that commonly shared items have been linked to outbreaks of MRSA. Evidence for the potential spread of MRSA via fomites comes mostly from the healthcare setting (5, 20, 33, 45) and from athletic teams and/or sports participants (9, 13, 17). Many advocate the importance of environmental sources of MRSA as potentially important contributors to MRSA acquisition in the healthcare setting (6, 16) while others cautiously agree that environmental reservoirs are potential sources of infection but point out gaps in the evidence for such spread (36). This argument carries over into the community setting, where there has been much less scientific investigation but where there are cycles of high public concern due to the occasional reports of MRSA infections in schools and athletes in the press, and because there is a potential for at least transient colonization of environmental surfaces and transfer of this pathogen, especially in institutional settings like prisons or schools, or other areas where many people may come into contact with common surfaces/fomites in rapid succession with variable amounts of cleaning between users.

C. Sampling Techniques

1. Sampling for pathogens from Environmental Surfaces

The recovery of microorganisms from environmental surfaces is known to be subject to a high degree of variability in both the reproducibility and sensitivity of the results (52). The effectiveness of any sampling method is influenced by many factors including those that contribute to the ability to remove the organisms from the surface,

the release of the organisms from the sampling device, and the subsequent detection of them via cultivation or other methods (50).

Environmental microbial sampling techniques for surfaces were first developed for the food safety industry and have thereafter been adapted to other fields such as the healthcare setting, industrial hygiene, and bioterrorism investigations. For the most part, the techniques used in these types of applications have remained largely the same since their inception: a swabbing based method followed by either direct platting or enrichment. To date, there is no accepted standard in microbiological surface sampling techniques either across fields or even within the same field; most methodologies are developed in-house by each user and are designed to meet their own specific needs.

This lack of standardization is due to many factors including poor overall recovery efficiencies across most methodologies, limitations in the types of surfaces that various methodologies are able to sample, the need to cater sampling and culture/enrichment techniques to specific organisms of interest, and a lack in the ability to adequately compare sampling methodologies across various studies due to various factors such as the reference surface materials used, sampling area, and surface matrix differences.

The two main schools of sampling methodologies in this field currently include swab-based methods in which a swab or other device is passed over a surface and then subsequently used to inoculate a culture plate or enrichment broth, or contact-based methods in which a device coated with growth media such as contact/Rodac plates or dip-slides are pressed directly onto a surface to remove organisms and directly incubated. There are obvious differences between these two approaches: e.g. contact-based methods

are currently only usable on hard, flat surfaces where swabbing techniques are more adaptable to irregular surfaces. Overall, contact methods have been shown to be the most effective in terms of recovery efficiencies and sensitivities compared to swab-based methods over a standardized sampling area (52) for *S. aureus* and *E. coli* when compared to swab-based approaches without an enrichment step; however, due to the limitations of contact-based methods in both the amount of surface area that can be sampled and their ability to be utilized on irregular surfaces, swab-base methods are still highly used in many environmental investigations in both research and industrial settings.

Laboratory Aspects	Real-World Aspects
Surfaces typically limited to hard, non-porous materials -glass, stainless steel, wallboard, polycarbonate, etc -devices may have variable efficiencies across surface types	**Surface Type Matrix Effects** -Depth -Composition -Porosity -Irregular surfaces
Seeding Concentrations -how do you compare trials which used dramatically different microbial seeding concentrations?	**Real-World Conditions/Processes** -physiochemical properties like humidity and temperature, etc -presence of dirt/grim
Individual Method Variables -type of swab, wetting agent, and processing steps were used and their individual effects	**User Mediated Variables** -Working Conditions -Individual Sampling Technique (rolling of swab buds and applied pressure)
Sampling Area -how much can a device sample? -what effect does this have?	
Molecular Effects -van der Waals forces -static electricity -contact angle -not well understood	

Table 8: Variables which confound comparisons between Environmental Surface Sampling Methods and their application in Real-World Settings (25)

Given the variables and difficulties in microbiological environmental surface sampling, the development of methods for these types of endeavors has been largely

addressed by each end-user (e.g. a hospital infection control team investigating potential environmental sources of healthcare-associated pathogens). Reported efficiencies for swab-based techniques have historically been low. In a report by Moore and Griffith, 2007, various factors influencing recovery using swab based methods including swab type and wetting agent were investigated. For *S. aureus*, recovery efficiencies from a directly inoculated stainless steel surface peaked at 8.9%; however, on a directly inoculated surface that had been allowed to dry for one hour, recovery across all swab/wetting agent combinations tested peaked at 1.4%. Mirroring these results work done by Obee *et al.* 2007, yielded roughly 1-10% efficiency for swab-based methods on a wet surface and a range of 0.07%-3.85% efficiencies from surfaces allowed to dry for 30 min after inoculation. Both of these studies were done on stainless steel coupons and are similar to results presented in other work. Reported recovery efficiencies for contact-based methods for *S. aureus* range from 38%-72.5% (52, 59).

As previously stated, although contact-based methods have been shown to yield higher recoveries, their limitations in the types of surfaces and the total area that can be used on currently limits their application to hard, flat, non-porous surfaces. The current state of this field means that other factors, besides sampling efficiency, have played a major role in the determination of which methods are commonly employed: cost, ease-of-use, suitability to the surfaces intended to be sampled, etc. This has produced a scene where there is some impetus to develop new technologies like vacuum-based collection or large scale wash methods, but no accepted standard as to how to compare any of these new or old methods such that each user must develop their own methods to fit their own limitations and needs.

D. Knowledge Gaps and Our Research Aims

The distribution of *Staphylococcus aureus* and MRSA in various environmental settings is poorly understood, especially community-based settings. Furthermore, the associated risks from environmental staphylococci are also unclear. Many of the questions that surround these issues stem from a lack of knowledge about how to recover these organisms from environmental sources, and more generally, a paucity of investigations of these pathogens outside of the healthcare setting.

In this investigation, we aimed to address some of these questions about the recovery of these pathogens from environmental surfaces, as well as gather data about the distribution of these organisms on environmental surfaces inside recreational exercise facilities; locations that have long been suspected of providing a high risk environment for environmental transmission of these pathogens.

We began our investigation by attempting to utilize methods previously used by other investigators to isolate these pathogens. Finding that common protocols used in past work investigating environmental surfaces were probably lacking in their ability to easily and reliably recover these organisms, we undertook our own investigation of sampling devices and other issues related to the recovery of these organisms. Eventually, we settled on a sampling device and protocol which we felt would provide reliable detection of these organisms and gathered data on the prevalence and levels of these pathogens on various surface types inside exercise facilities. We hope that the information garnered from our investigation is useful in the development of risk

assessment and management steps for *S. aureus* and MRSA inside these types of facilities as well as other types of settings.

VI. Experimental Design

This work was done in three experimental phases. The first phase was an evaluation of the bacterial loading on the target sampling surfaces inside exercise facilities to evaluate potential sampling interference by background organisms present on surfaces. These data were used to select candidate methods for sampling procedure, enrichment culture, and colony isolation and identification media. The second phase was a comparison of candidate sampling and plating methods. Based on the comparison conducted in Phase 2, a method was selected for use in Phase 3. Phase 3 consisted of environmental sampling conducted at a number of exercise facilities to determine the presence and levels of MRSA on surfaces.

VII. Materials and Methods

A. Phase1-Evaluation of Background Bacterial Contamination of Target Surfaces

Polyester swabs were used to sample 100 cm^2 areas (10cm x 10cm) on each of 10 surfaces at a local exercise facility. Surfaces sampled are listed in Table 9.

[illegible]	[illegible]
1	Free-weight Handle
2	Dip-Assist Platform Handle
3	Free-weight Bench
4	Free-weight Bench
5	Barbell Handle
6	Barbell Bench
7	Elliptical Machine Handle
8	Butterfly Machine Bench
9	Exercise Mat
10	Medicine Ball

Table 9: List of Samples taken for Background Bacterial Contamination Investigation

Each swab was wetted by immersion in 1.5mL of TSB (Trypticase Soy Broth) in a 10mL polystyrene tube, the excess liquid was expressed by pressing and rolling the

swab around the rim of the tube, and then the wetted head of this swab was applied to the sample area with moderate pressure and passed in a tight, zig-zag pattern across the entire sampling surface in two perpendicular directions. The head of the swab was rolled during the sampling process. After sampling, the swab was returned to the polystyrene tube and broken off into the liquid. All samples were returned to lab within one hour of sampling. Tubes were vortex mixed and then 0.5mL of each sample was spread plated on both mannitol salt agar (MSA) and sheep's blood agar (SBA). The remaining liquid samples were incubated at room temperature for 24 hours, and two 0.25 mL volumes were spread plated on MSA and SBA. All plates were incubated for 24 hours at 35°C. After incubation, all plates were examined for growth, colonies were counted and presumptive identification of bacterial classes was made. Colonies typical of *Staphylococcus aureus* were subcultured to TSA (Tryticase Soy Agar) and then resultant colonies were picked and emulsified in deionized H_2O on a glass slide which was left to dry, heat fixed and then gram-stained, catalase tested, and tested for coagulase production via the Staphylase Test kit (Oxoid).

B. Phase 2-Comparison of Candidate Sampling Protocols

1. Sampling Device Evaluation-Representative Surfaces

Sampling Devices Analyzed

- Polyester Swabs-(Fisherbrand) polyester fiber spun around a plastic shaft.
- Macrofoam Swab-(VWR) foam head on a plastic shaft.
- Gauze Wipe-(Versalon) 4" x 4" polyester-rayon blend wipe.

Sampling Surfaces

For evaluation of sampling methods, test surfaces representative of those found in exercise facilities were used. Surfaces tested were:

- Smooth Metal Bar-Handle of a 2lb CAP brand dumbbell purchased from Play It Again Sports (7.23cm circumference x 6.92cm length). All dumbbells used were new when purchased.

- Textured Metal Bar-Cut sections of a cross-textured handle of a used barbell purchased from Play It Again Sports (8.17cm circumference x 6.11cm length). The barbell sections had various levels of weathering from use but were typical of those found in many exercise facilities.

- Vinyl-Square pieces affixed to wooden backing. Sheets of Vinyl purchased from Stewart Macdonald (http://www.stewmac.com/) Item #1656-Black Vinyl covering. The vinyl was typical of that found on gym equipment as covering for mats and benches and was moderately textured. (7.07cm x 7.07cm)

- Rubber-Square pieces of foam rubber affixed to wooden backing. Constructed from rubber grip tape commonly used on tennis and racquetball racquets (Wilson Pro Soft Overgrip). This material modeled the material commonly used on hand-grips of exercise equipment. (7.07cm x 7.07cm)

Both types of metal bars were wrapped and autoclaved to sterilize them between experimental runs. The Vinyl and Rubber surfaces were sprayed and saturated with 70% ethanol and exposed to germicidal UV for 30 minutes to sterilize them between runs.

Experimental runs were conducted starting with the smallest bacterial inoculum and working to the largest to minimize any potential carryover effects.

Bacterial Inocula for Recovery Experiments

The method for creating bacterial inocula was adapted from Environmental Protection Agency (EPA) method 1601 (72). A frozen stock of *Staphylococcus aureus* ATCC strain 25923 was used to inoculate 25mL of TSB, which was incubated in a shaker flask at 100rpm at 35°C. After 4 hours, the optical density at 520 nm was measured. When the OD reading was at between 0.1-0.5, the culture was centrifuged at 7,277xg for 10 minutes at 4°C and the pellet was resuspended in 25mL of PBS (Phosphate Buffered Saline). Serial 10-fold dilutions of the bacterial suspension were then prepared in PBS and the suspension was titered by spread-platting 100µL of each dilution onto 3 replicate TSA plates which were then incubated for 24 hours at 35°C. The bacterial suspensions were then held at 4°C for no more than 24 hours before use.

Inoculation of Test Surfaces

The bacterial suspension was diluted with PBS to the target concentration for each experimental run. The inoculum was delivered in 25 2µL drops distributed evenly over a $50cm^2$ test surface. Test surfaces were left to dry on the bench at room temperature for 2 hours before sampling.

Sampling Procedure

For swab sampling, 0.85% NaCl was used as the wetting agent. Each swab was submerged into 1mL of the wetting agent inside a glass test tube and expressed by pressing and rolling the swab head around the rim of the tube. The test surface was sampled by passing the swab back and forth with moderate pressure on the surface in a

tight, zig-zag pattern in two perpendicular directions while rolling the head of the swab. The swab was then broken-off into the test tube and stored at 4°C for 1 hour.

Gauze wipes were folded into 1'' x 1'' squares and then wetted with 1mL of 0.85% NaCl. The wipe was then passed over the test surface in a tight, zig-zag pattern applying moderate pressure in two perpendicular directions with a new face of the wipe being used during the second direction of sampling. The wipe was then unfolded and placed into 29mL of the wetting agent in a 50mL conical polystyrene tube and held at 4°C for 1 hour.

Sample Processing

All swabs were vortex mixed for 10-20 seconds and 100µL of the suspension was spread plated on TSA. Wipes were vortex mixed for 20 seconds, the wipe removed and the liquid expressed back into the sample tube containing the released bacteria. The sample tube was then centrifuged at 7,277 xg for 10 minutes and all but 1 mL of the supernatant removed. The bacterial pellet was then resuspended via vortex mixing and 100µL was spread plated on TSA. All plates were incubated for 24 hours at 35°C and then examined for colony growth and their enumeration. There were 3 replicates for each sampling device, inoculum concentration, and surface type combination. Inocula concentrations were confirmed by spread plating serial dilutions on TSA, incubating and counting resulting colonies.

Analysis

Expected colony counts on each sample plate were calculated from the initial inoculum titer. For example, if we obtained an original titer of 1000cfu/50µL of inoculum and 50µL of incoulum was placed on each test surface we would expect ~1000cfu to be present on the surface. If all inoculated cfus were recovered and then placed into 1mL of

liquid, we would expect to detect approximately 10% of the inoculated and recovered cfus (i.e. 100cfus) if we platted 100μL of the liquid. These expected plate counts were compared to actual plate counts to calculate recovery percentages.

2. Survival Assay of *S. aureus* on Metal Coupons

Inoculum volumes of 50μL containing ~1,000cfu of *S. aureus* suspended in PBS were inoculated onto 1'' x 1'' metal (stainless steel) coupons in 25 2μL drops (~1150cfu/50μL inocula for run 1 and ~1500cfu/50μL in run 2). Three replicates were done per experiment. All coupons were placed in sterile plastic petri dishes and left to dry for 0, 30, 60, 90, or 120 minutes. At each time point, one set of coupons was eluted using 5 mL of a wetting agent (0.85% saline or PBS) added to the petri dish and agitation on a shaker platform at 60rpm for 20 minutes. After elution, the wetting agent was aspirated and centrifuged at 7,277 xg for 10 minutes at 18°C. After centrifugation, the top 4mL of supernatant was removed, the bacterial pellet resuspended in the remaining volume, and 10-100μL aliquots of each resultant suspension was spread plated on TSA. Plates were incubated for 48 hours at 35°C. A total of 6 trials for each sampling time were conducted for both runs 1 and 2. Plate counts were compared to expected plate counts based on titer of the original inoculum to calculate recovery percentages.

3. Resuspension Media Assay

A suspension of *S. aureus* (see "Bacterial Inocula for Recovery Experiments" above) was diluted to approximately 6000cfu/50µL. 50µL of the inoculum was placed onto 1'' square stainless steel coupons inside petri dishes and spread evenly over the coupon surface with a disposable plastic cell spreader. The coupons were allowed to dry for 2 hours and 5mL of a test recovery medium (PBS, PBS with 0.1%Tween 80, Dulbecco's PBS, or Dulbecco's PBS with 0.1% Tween 80) was added to the petri dish. All petri dishes were agitated on a shaker platform at 60rpm for 20 minutes. After agitation, the recovery medium from each petri dish was placed into a 10 mL polystyrene tube, centrifuged, (4°C, 10 minutes, 7,277 xg), the top 4 mL of media removed and the bacterial pellet resuspended in the resultant 1 mL of remaining medium. The resuspended organisms were spread plated on TSA (100µL on 10 replicate plates) and counted at 24 and 48 hours of incubation at 35°C. Each recovery medium was tested in triplicate.

4. Recovery Media Assay

A suspension of *S. aureus* (see "Creation of Bacterial Inoculum" above) was diluted to approximately 6000cfu/50µL. A 50µL volume of the inoculum was placed onto 1'' square stainless steel coupons inside petri dishes and spread evenly over the coupon surface with a disposable plastic cell spreader. The coupons were allowed to dry for 2 hours and 5mL of a test recovery medium (PBS, PBS with 0.1%Tween 80, Dulbecco's PBS, or Dulbecco's PBS with 0.1% Tween 80) was added to the petri dish.

All petri dishes were agitated on a shaker platform at 60rpm for 20 minutes. After agitation, the recovery medium from each petri dish was placed into a 10 mL polystyrene tube, centrifuged, (4°C, 10 minutes, 7,277 xg), and resuspended in 1 mL. The resuspended organisms were spread plated on TSA and resulting colonies were counted at 24 and 48 hours of incubation at 35°C. Each recovery medium was tested in triplicate.

5. Recovery from Sampling Device Assay

A bacterial suspension of *S. aureus* (see "Bacterial Inoculum for Recovery Experiments" above) was diluted to ~2000cfu/100µL in PBS. A 100µL volume of this new suspension was applied directly to the candidate sampling devices (polyester swabs, macrofoam swabs, 1''x1'' polyester-rayon blend gauze wipes) which were either dry (run 1) or pre-wetted with Dulbecco's PBS with 0.1% Tween 80 (run 2). After applying the inoculum to the sampling device, the device was transferred to a 10mL polystyrene tube containing 2mL of Dulbecco's PBS and 0.1% Tween 80. Controls were performed at this step by directly inoculating tubes containing 2mL of Dulbecco's PBS with 0.1% Tween 80 with 100µL of the test inocula. All tubes were vortex mixed at maximum setting for 20 seconds and spread plated on TSA. Plates were incubated for 24 hours at 35°C. Mean colony counts obtained from each trial were compared to the mean colony counts from the control to determine retention of *S. aureus* on sampling devices.

6. Sampling Device Evaluation-Hard, Flat, non-porous Surface

Sampling Devices Analyzed

- Polyester Swabs-(Fisherbrand) polyester fiber spun around a plastic shaft.
- Macrofoam Swab-(VWR) foam head on a plastic shaft.
- Gauze Wipe-(Versalon) 4'' x 4'' polyester-rayon blend wipe.
- Swiffer™ Wipe-(Swiffer™ Brand Dry Sweeping Cloths) Electrostatic Wipes

Inoculation of Test Surfaces

A 4-hour culture of *S. aureus* in TSB was titered and diluted in PBS to the desired inoculum concentration. In some experimental runs, sterile defibrinated sheep's blood was added (5% v/v) to mimic organic loading on surfaces. A 100µL volume of the inoculum was applied to 10cm x 10cm stainless steel coupons in plastic petri dishes, and the inoculum was spread evenly over the sampling surface with a plastic disposable cell spreader. The test surfaces were allowed to dry for 2 hours at room temperature.

Sampling Procedure

For Swabs: Swabs were moistened with Dulbecco's PBS with 0.1% Tween 80 and then used to sample a test surface by passing the swab, while rotating the head of the swab and applying moderate pressure, in a tight zig-zag pattern in two perpendicular directions over the test surface. The swabs were then broken off into 2mL of Dulbecco's PBS with 0.1% Tween 80 in a 10mL polystyrene tube.

For Wipes: Wipes were cut down to approximately 1'' squares before use. Wipes were moistened with Dulbecco's PBS with 0.1% Tween 80 (100µL) and, with moderate pressure, used to sample the test surfaces by passing the wipe in a tight zig-zag pattern in two perpendicular directions. A new face of each wipe was used to sample the test

surface in the second direction. After sampling, each wipe was placed into 2mL of Dulbecco's PBS with 0.1% Tween 80 in a 10mL polystyrene tube.

Controls: 100µL of the bacterial inoculum was pipetted into 2 mL of Dulbecco's PBS with 0.1% Tween 80 in a 10 mL polystyrene tube.

Sample Processing

Samples remained at room temperature for 30 minutes and were then vortex mixed at maximum setting for 20 seconds to release bacteria into the Dulbecco's PBS with 0.1% Tween 80. Samples were spread plated on TSA (100µL/plate) and incubated for 24-48 hours at 35°C. Colonies were counted and sampling efficiency was calculated by comparison of colony concentrations of samples to concentrations of inoculum controls.

Data Analysis

Sampling efficiencies were calculated by comparing mean colony counts from each trial set to the mean colony counts in the control sets. In an effort to reduce the effects of within trial outliers, a 20% Trimmed Mean method (78) was also used in this evaluation to exclude the extreme values in each set of plate counts and then the mean from these sets of data used to calculate recovery efficiency in the same manner.

7. Sampling Device Evaluation using a Most Probable Number Enrichment Method

Sampling Devices Analyzed

- Polyester Swabs-(Fisherbrand) polyester fiber spun around a plastic shaft.

- Macrofoam Swab-(VWR) foam head on a plastic shaft.
- Gauze Wipe-(Versalon) 4'' x 4'' polyester-rayon blend wipe.
- Swiffer™ Wipe-(Swiffer™ Brand Dry Sweeping Cloths) Electrostatic Wipes

Inoculation of Test Surfaces

A 4 hour culture of *S. aureus* in TSB with 5% v/v sterile defibrinated sheep's blood was used as the inoculum in this experiment. Volumes of 100μL of the inoculum were spread with a disposable plastic cell spreader over the surface of 10cm x 10cm stainless steel coupons. A 100μL volume of the inoculum was also added directly to 6 mL of DPBS with 0.1% Tween 80 as a control. All inoculated surfaces were allowed to dry for 30 minutes.

Sampling Procedure and Processing

The MPN procedure was adapted from the US Food and Drug Administration Bacteriological Analytical Manual (2). Sampling was carried out as describe above, except that sampling devices were placed into 6mL of DPBS with 0.1% Tween 80 in a 10mL polystyrene tube. Samples were held for 10 minutes before being vortex mixed at maximum setting for 20 seconds. One mL of each sample was then used to create 10-fold serial dilutions from 10^{-1} to 10^{-9} in PBS. These sample dilutions were used to inoculate a 5 tube MPN assay by adding 1mL volumes of the appropriate dilution to 9mL volumes of TSB amended with 10% NaCl and 1% Na pyruvate. Tubes were incubated at 35°C for 48 hours and tubes exhibiting cloudy growth were scored as positive. The Most Probable Number (MPN) was calculated according to the MPN tables in the Bacteriological Analytical Manual using the results of 3 dilutions where the highest dilution contained 0 positive tubes

C. Phase 3-Environmental Sampling

1. Environmental Sampling of Exercise Facilities

Recruitment of Exercise Facilities

Internet and phone book searches of local gyms and exercise facilities in Orange, Wake, and Durham Counties (NC) were conducted and potential sites for sampling identified. Potential sites were contacted via email, phone calls, and/or mail to elicit participation in the study. Institutional Review Board approval was unnecessary as no direct interaction with individuals needed to occur. Sites received a form letter explaining the background and purpose of the study and were promised that their results would be reported to them but otherwise kept confidential (See Appendix). Sites were also offered recommendations for additional cleaning/protective actions against *S. aureus* and MRSA upon their request.

Approximately 50-75 facilities were contacted. Eight facilities responded that they would be willing to participate in the study. The majority of facilities contacted did not return attempts to speak to a manager or owner via phone or email. Two of the facilities that were contacted specifically indicated that they did not wish to participate citing potential implications of study findings and negative publicity as major concerns. A basic description of each site included in the study is presented in Table 10 below; all sites were Co-ed except for site 3, which was a women's only facility. All sites had a set cleaning protocol except for site 5; all other sites each used a different cleaning agent(s). Sites 1 and 2 had disinfectant wipes available for patrons to use to wipe down equipment themselves. In addition, site 1 as well as site 8 had cleaning agents in spray bottles and cleaning rags available for patrons to use as they saw fit. Specific adherence to cleaning

regimes or patron use of cleaning agents was not investigated. It was originally planned to incorporate about the same number of each type of Site into this investigation, but the low number of respondents and time limitations prevented this from being achieved.

Site Number	Number of Samples Collected	Site Type	Site Location	Mean Patron Age
Site 1	50	Large University Facility	Orange	18-25
Site 2	59	Large Corporate	Orange	35-45
Site 3	26	Personal Trainer	Durham	45-55
Site 4	41	Personal Trainer	Wake	25-35
Site 5	16	Apartment Complex Facility	Durham	25-35
Site 6	20	Personal Trainer	Durham	35-45
Site 7	30	Personal Trainer	Durham	18-25
Site 8	50	Large University Facility	Wake	18-25

Table 10: Description of each Exercise Facility included in field sampling

Environmental Sampling of Surfaces in Gym/Exercise Facilities

Sampling surfaces consisted of various types of exercise equipment, although other surfaces within these facilities were also sampled (e.g. door handles, locker room benches). Surfaces to be sampled were convenience samples and chosen based on relative numbers and types of equipment pieces found within the facility. The number of samples obtained from each facility was dictated based upon available man-power to process specimens at the laboratory, the amount of time researchers were allowed to sample at each facility (typically no more than 1.5 or 2 hours) and the amount of equipment within each facility. Those facilities that were larger represent a larger number of the total samples taken. Patrons were not asked to cease activity for sampling purposes. Samples were collected by spraying Dulbecco's PBS with 0.1% Tween 80 onto

the surface with a spray bottle. A polyester-rayon blend gauze wipe was passed over the entire surface in a tight zig-zag pattern with moderate pressure, folded to expose a new face of the wipe, and then passed in the same fashion over the surface in a perpendicular direction. In the case of irregular surfaces such as bars or handles, the wipe would be wrapped around the handle, gripped with moderate pressure, and twisted back and forth about the surface as well as side-to-side. Sample wipes were immediately placed into 90 mL of TSB amended with 10% NaCl and 1% Na pyruvate in a 125mL polypropylene bottle and the bottles stored in a chilled cooler. Samples were transported to the lab and processed within 2 hours of collection. The following information regarding the surfaces was recorded: descriptions of the type of exercise equipment sampled, probable body contact sites with which the surface is associated (either the hands or the body/torso), and the type of exercise with which the surface/equipment is associated.

Samples from various surfaces were grouped for analysis based upon the type of exercise with which the surface was associated: Strength Training, Aerobics, Calisthenics, or Other. Surfaces associated with "Strength Training" were those associated with the movement of weights of some type (e.g. exercise machines that provided moderate to heavy resistance, free weights, barbells, medicine balls, etc. and any dedicated bench surfaces associated with these items: e.g. benches dedicated for use with free weights or barbells). Surfaces associated with "Aerobics" included those items used in activities associated with Aerobic exercise: treadmills, exercise bikes, ellipticals, jump ropes, etc. Surfaces associated with "Calisthenics" included those surfaces where outside weight was not introduced for additional resistance in the exercise (i.e. only body weight was used) and included surfaces associated with push-ups, stretching, yoga, sit-

ups/crunches, and other exercises of this nature. Surfaces grouped into the "Calisthenics" group included items such as stretching mats, exercise balls, yoga mats, benches designed for sit-ups, and equipment designed only for un-assisted leg raises. Surfaces such as locker room benches, shower heads/handles, and door handles not associated with actively exercising were categorized as "Other." A breakdown of the samples collected from each site based upon the exercise and body contact site of each sample is presented in Table 11 below.

Type of Exercise	Aerobics	Calisthenics	Strength	Other	Totals
Site 1	14 (10/4)	6 (2/4)	20 (13/7)	10 (6/4)	50 (31/19)
Site 2	19 (14/5)	8 (0/8)	32 (19/13)	0 (0/0)	59 (33/26)
Site 3	0 (0/0)	0 (0/0)	24 (13/11)	2 (2/0)	26 (15/11)
Site 4	0 (0/0)	4 (0/4)	35 (23/12)	2 (2/0)	41 (25/16)
Site 5	5 (4/1)	0 (0/0)	11 (4/7)	0 (0/0)	16 (8/8)
Site 6	0 (0/0)	4 (0/4)	14 (13/1)	2 (2/0)	20 (15/5)
Site 7	4 (4/0)	6 (0/6)	20 (14/6)	0 (0/0)	30 (18/12)
Site 8	8 (6/2)	7 (1/6)	35 (21/14)	0 (0/0)	50 (28/22)
Totals	50 (38/12)	35 (3/32)	191 (120/71)	16 (12/4)	292 (173/119)

Table 11: Number of Samples collected from each site based upon Exercise Type with which the surface is associated. Presented as Total Number of Samples (Samples associated with Hand Contact/Samples associated with Torso Contact)

Sample Processing

Samples were vortex mixed at maximum setting for 1 minute to release bacteria into the broth. A 3-dilution, 5 tube MPN method was used to measure *S. aureus* levels in each sample by placing 10mL, 1mL, and 0.1mL volumes into 5 replicate tubes of TSB with 10% NaCl and 1% Na pyruvate. The MPN tubes and original sample bottle (with the remaining broth and sampling wipe inside) were incubated for 48 hours at 35°C. After incubation, a sterile bacteriological loop was used to transfer a small amount of broth from each tube in the MPN assay and each original sample bottle to a section of a Baird-Parker agar plate. Each plate was incubated at 35°C for 48 hours.

Identification and Enumeration of S. aureus

The inoculated Baird-Parker agar plates were examined for bacterial colonies typical for *S. aureus* (black colonies with a translucent halo) and up to 3 colonies from each sample set (the MPN tubes and sample bottle for each sample) were sub-cultured to MSA. These isolates were then tested via gram staining, catalase reaction, and tube coagulase (Rabbit Plasma with EDTA-Becton Dickenson). Isolates that were identified as *S. aureus* were sub-cultured to a tube of TSB broth with 20% glycerol and stored frozen at -80°C. If a sample set was found to have at least one isolate that was identified as *S. aureus*, then plate sections that had typical colony morphology for *S. aureus* were scored as positive for *S. aureus*. The MPN was calculated using tables from the Bacteriological Analytical Manual (4). The MPN values obtained from the table were adjusted to match the total sample volume of each sample bottle, by dividing by 10 to yield results per 10mL and multiplying by 9 to yield results for 90mL (total sample volume). Values below the detection limits of this assay were assigned a value of 1 cfu/sample and those above the detection limits assigned a value of 1440 cfu/sample (Detection limits of the assay were: lower detection limit 1.62 CFU/sample, upper detection limit 1440cfu/sample).

Identification of MRSA

S. aureus isolates were tested for methicillin resistance by adjusting a suspension of the isolate to a 0.5 McFarland Standard, placing 10μL on BD™ Oxacillin Screen Agar, and incubating at 35°C for 24 hours. Any growth was scored as positive for MRSA. MRSA isolates were sent for Pulsed-Field Gel Electrophoresis typing

performed by Maria Gergin (MT-ASCP) of the Epidemiology Department of UNC Hospitals. The written procedure for the PFGE analysis is presented in the Appendix.

2. Time-Series Analysis of *S. aureus*/MRSA loading on Gym Equipment

Ten surfaces at a previously sampled exercise facility were chosen for analysis: 5 barbell benches, and 5 bench surfaces from various exercise machines. Samples were taken twice a day at 10am and 3pm on 3 days (Wednesday, Thursday, and the following Monday) and processed/analyzed as described in "IX: Environmental Sampling of Exercise Facilities." Levels and prevalence of *S. aureus* and MRSA were compared across these repeated samplings. MRSA isolates were sent for PFGE analysis at UNC Hospitals as described above in "*Identification of MRSA*" in "Environmental Sampling from Exercise Facilities."

VIII. Results

A. Phase I-Evaluation of Background Bacterial Contamination of Target Surfaces

All 10 of the surfaces tested for background bacterial contamination showed growth. All samples had colonies with morphology consistent with *Staphylococcus* or *Streptococcus* species on SBA. Presumptive *Staphylococcus* or *Streptococcus* colony counts ranged from 2 to 150 CFU per SBA plate. Additionally, all but one sample had other types of organisms growing on SBA: 1 sample had yeast, 1 had gram negative rods, 4 had one or more *Proteus* species swarming much of the plate, and 6 had one or more *Bacillus* species covering much of the plate. All but one sample had growth on MSA. Colony counts on MSA ranged from 0 to 100, and 6 samples had colonies consistent with Staphylococcal species. Of the samples obtained, only 2 had *Staphylococcus aureus* present.

B. Phase II-Comparison of Candidate Sampling Methods

1. First Permutation of Sampling Device Evaluation-Representative Surfaces

This evaluation of candidate surface sampling procedures was performed in an attempt to assess the recovery of candidate sampling devices on surface types expected to be encountered during environmental sampling. A range of inocula were tested, and with an inoculum of ~655 cfu, sampling efficiency ranged from 0 to 57.5% across all sampling

device/surface combinations (Table 12). Mean sampling efficiencies for were 4.7% for polyester swabs, 13.9% for macrofoam swabs, and 8.0% for polyester-rayon blend gauze wipes. However, high variability was observed within each group (Figures 1-4).

Individual

	Polyester Swab	Macrofoam Swab	Gauze Wipe
Vinyl	6.1	4.6	2.5
Rubber(Flat)	1.0	57.5	31.0
Rubber(Curved)	0.0*	3.1	4.6
Metal(Smooth)	16.3	0.0*	1.0
Metal(Textured)	0.0*	4.6	1.0

Table 12: Mean Sampling Efficiencies (%) of each sampling device on each representative surface at ~655 cfu/surface inoculations (n=3 for each data point) (* indicates no colonies grew on inoculated TSA plates for any replicate)

Device	Surface	Recoveries (%)		
		Trial 1	Trial 2	Trial 3
Polyester Swab	Vinyl	4.6	10.7	3.1
	Rubber(Flat)	1.5	1.5	0.0
	Rubber(Curved)	0.0	0.0	0.0
	Metal(Smooth)	4.6	33.6	10.7
	Metal(Textured)	0.0	0.0	0.0
Macrofoam Swab	Vinyl	1.5	6.1	6.1
	Rubber(Flat)	7.6	29.0	135.9
	Rubber(Curved)	0.0	7.6	1.5
	Metal(Smooth)	0.0	0.0	0.0
	Metal(Textured)	0.0	12.2	1.5
Polyester-Rayon Blend Gauze Wipe	Vinyl	4.6	1.5	1.5
	Rubber(Flat)	10.7	80.9	1.5
	Rubber(Curved)	10.7	3.1	0.0
	Metal(Smooth)	0.0	1.5	1.5
	Metal(Textured)	0.0	1.5	1.5

Table 13: Sampling efficiencies (%) from each trial replicate for each sampling device and representative surface combination at ~655cfu/surface inoculation (0.0 indicates no detectible recovery)

Variability was observed during these experiments, especially at lower inocula levels (1cfu and 9.2 cfu/surface). When low titer inocula were used, some inconsistent results were observed: mainly colony counts that were several orders of magnitude higher than the original inoculum. These results were often from unexplained high numbers of bacterial for one replicate within each sampling group.

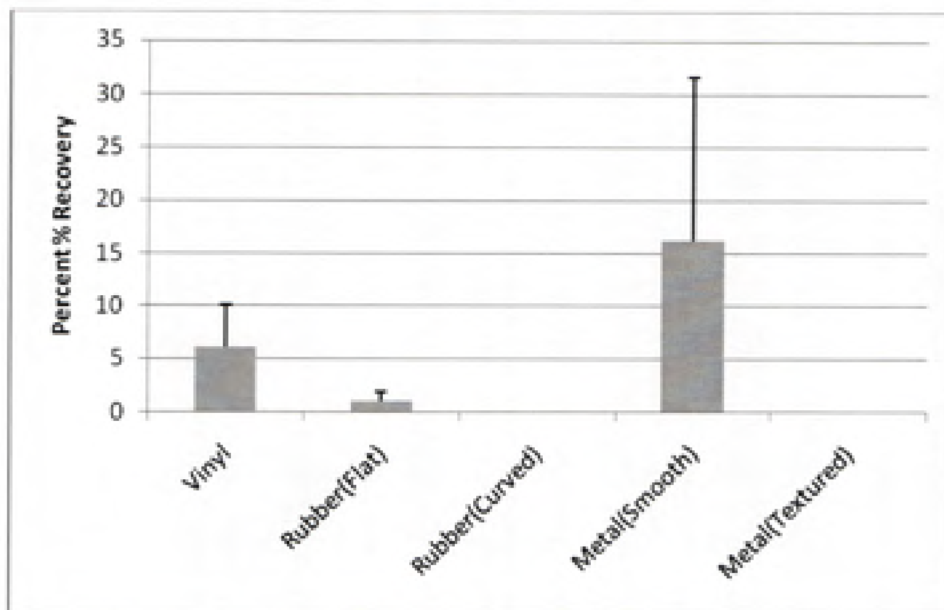

Figure 1: Sampling Efficiency of Polyester Swabs on each surface type at ~655cfu inoculations (error bars are 1 Standard Deviation)

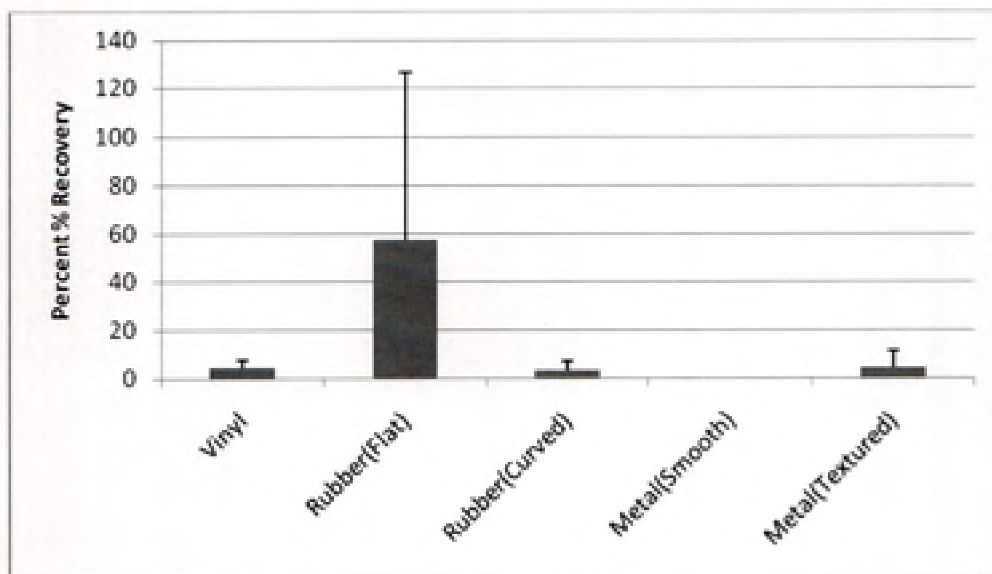

Figure 2: Sampling Efficiency of Macrofoam Swabs on each surface type at ~655cfu inoculations (error bars are 1 Standard Deviation)

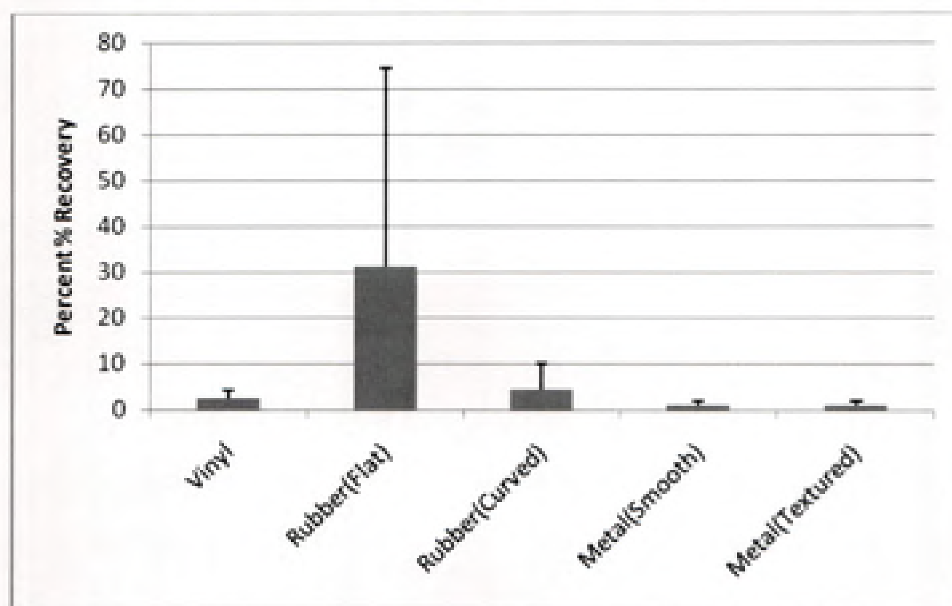

Figure 3: Sampling Efficiency of Polyester-Rayon Blend Gauze Wipes on each surface type at ~655cfu inoculations (error bars are 1 Standard Deviation)

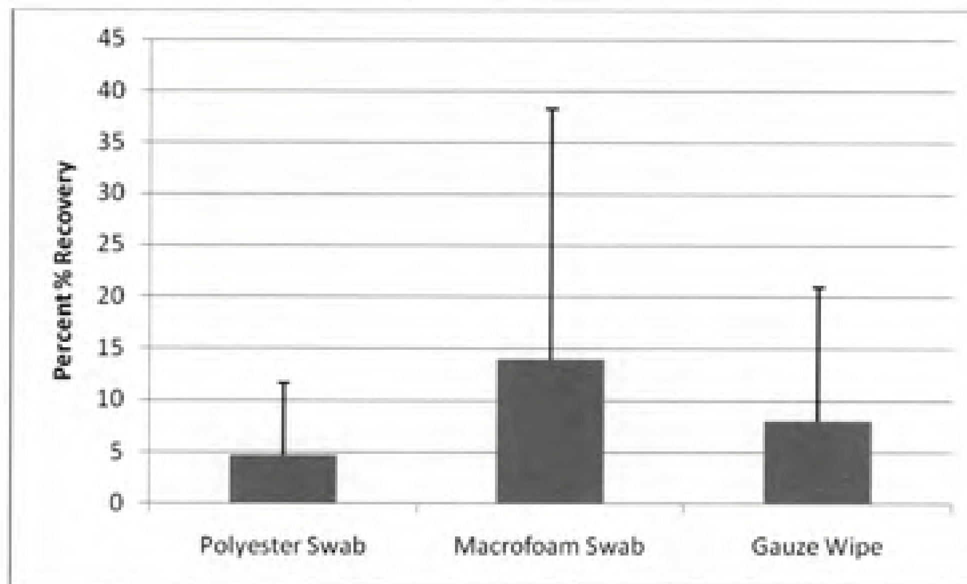

Figure 4: Mean Sampling Efficiency (%) of each sampling device across all surfaces (error bars are 1 Standard Deviation)

Overall results suggest that recovery efficiencies are low for all sampling devices and surface types evaluated. A series of experiments was performed to determine which steps of the sampling protocol were the sources of bacterial losses. Later evaluations of candidate sampling devices utilized an ideal surface to minimize variability in sampling recovery and bacterial viability between trials.

2. Survival of *S. aureus* on Metal Coupons

Experiments were conducted to determine if low recovery efficiencies of viable bacteria were due to inactivation or irreversible binding of bacteria on surfaces in sampling experiments. Losses in viable recoverable bacteria were observed in these experiments. The first trials of this experiment were conducted with 0.85% saline as the eluting agent. The number of replicates at each time point was 6 (Figure 5).

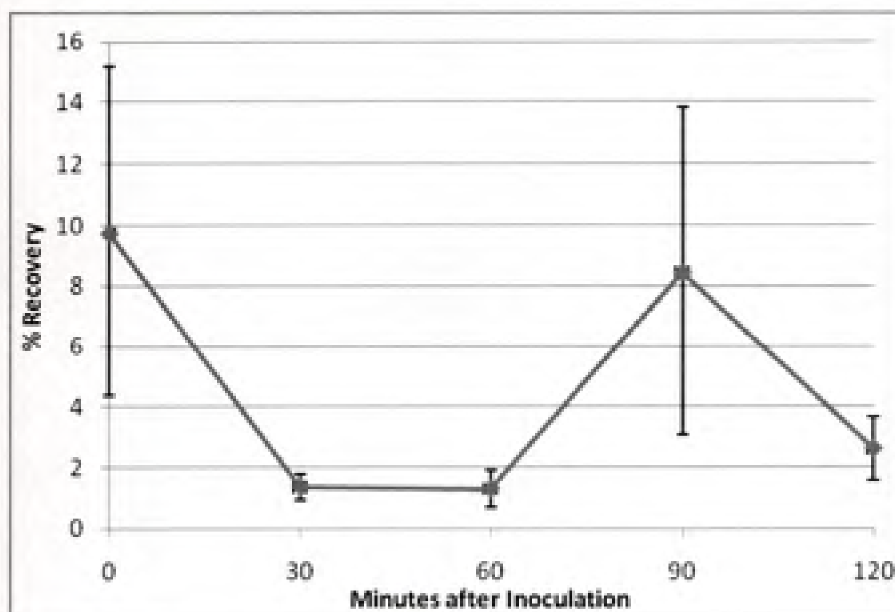

Figure 5: Mean Recovery (%) of S. aureus inoculated onto stainless steel coupons and sampled at 30 minute intervals with 0.85% saline as the eluting agent(n=6 for each time point, error bars are 95% Confidence Limits)

Apparent increases in bacteria recovery also occurred in later trials of this experiment in which PBS was used as the eluting agent. A time series recovery experiment is shown in Figure 6. In both sets of experiments, some viable bacteria in the inoculum seem to become undetectable as soon as they are deposited onto the test surfaces. It is unclear whether this is due to die-off, lack of culturability, injury, unrecoverability from the inoculated surface or physical loss of cells during subsequent processing steps. It appears that there is a further loss of viable bacterial cells over time as drying occurs (an additional approximately 80-90%), especially during the first hour, after which bacterial numbers do not seem to decline measurably between 60 and 120 minutes.

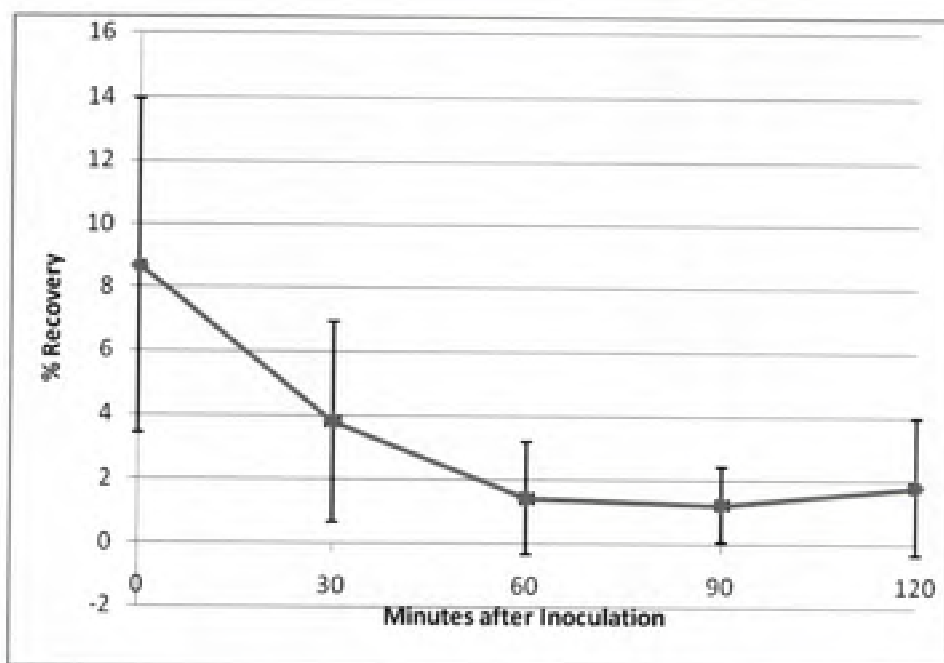

Figure 6: Mean Recovery (%) of S. aureus inoculated onto stainless steel coupons and sampled at 30 minute intervals with PBS as the eluting agent (n=6 for each time point, error bars are 95% Confidence Limits)

3. Resuspension Media Evaluation

Percent Survival at 24hours	
PBS	100.2-102.7%
PBS w/ 0.1% Tween 80	101.2-102.7%
Dulbecco's PBS	100.7-102.5%
Dulbecco's PBS w/ 0.1% Tween 80	98.3-101.8%

Table 14: Survival of *S. aureus* in candidate resuspension media at 4° C for 24 hours (original inoculum ~7 log_{10})

An experiment was done to evaluate potential effects of various media used to resuspend and store bacterial cells on their viable cell concentrations. Over a 24 hour period, no losses in viable *S. aureus* were observed in any of the tested media (Table 14). Initial inoculum concentrations were 6.5, 6.8, and 7.1 log_{10}. There were no statistically significant differences in quantities of recovered cells between the media.

4. Recovery Media Evaluation

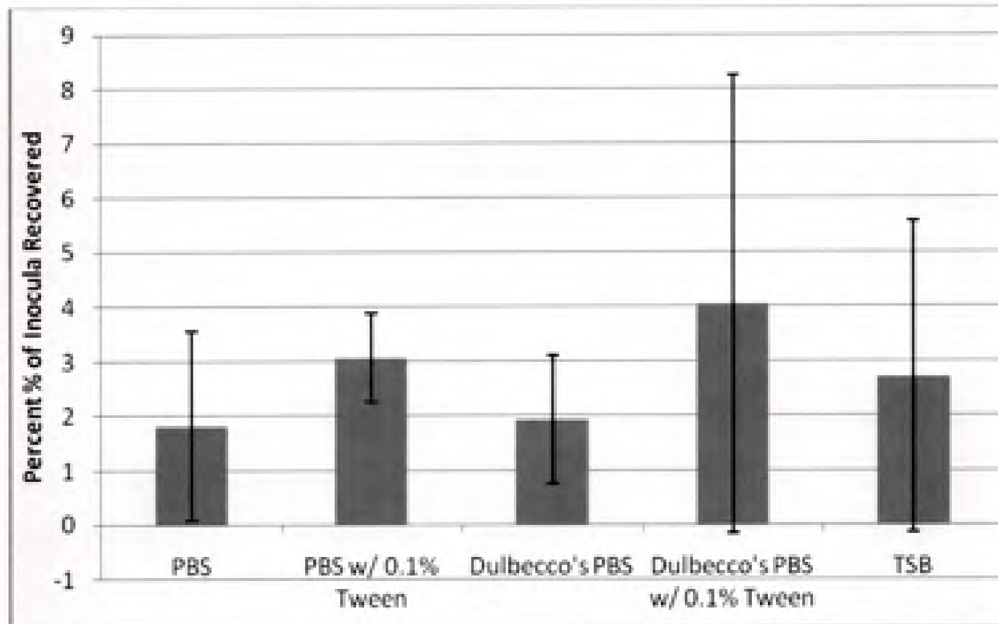

Figure 7: Recovery of *S. aureus* inoculated with ~6200 cfu by various eluting media (n=3, error bars are 95% Confidence Intervals)

This experiment was intended to determine the effectiveness of various media as wetting agents to release bacterial cells from test surfaces. Media with 0.1% Tween had overall higher mean recoveries, although there were no statistically significant differences in percentage of inoculated cells recovered between the recovery media tested.

5. Evaluation of Recovery from Sampling Devices

An evaluation was performed to examine the ability of candidate sampling devices to release bacterial cells once they had been taken up into the sampling devices under the anticipated conditions for environmental sampling: immediate placement of the sampling device into some liquid medium for transport to the laboratory. Thus, the

inoculum placed on each sampling device was not allowed to dry; rather, the sampling devices were immediately placed into Dulbecco's PBS with 0.1% Tween 80 after bacterial inocula were applied. One replicate of the Swiffer™ wipes had much lower recovery than the other two runs, resulting in a very large confidence interval (Figure 8). Pre-moistening with DPBS with 0.1% Tween 80 did not change the amount of bacterial recovery (Figure 9). There was no statistical difference based upon 95% confidence intervals about each point compared across sampling devices or between non-prewetted and prewetted devices.

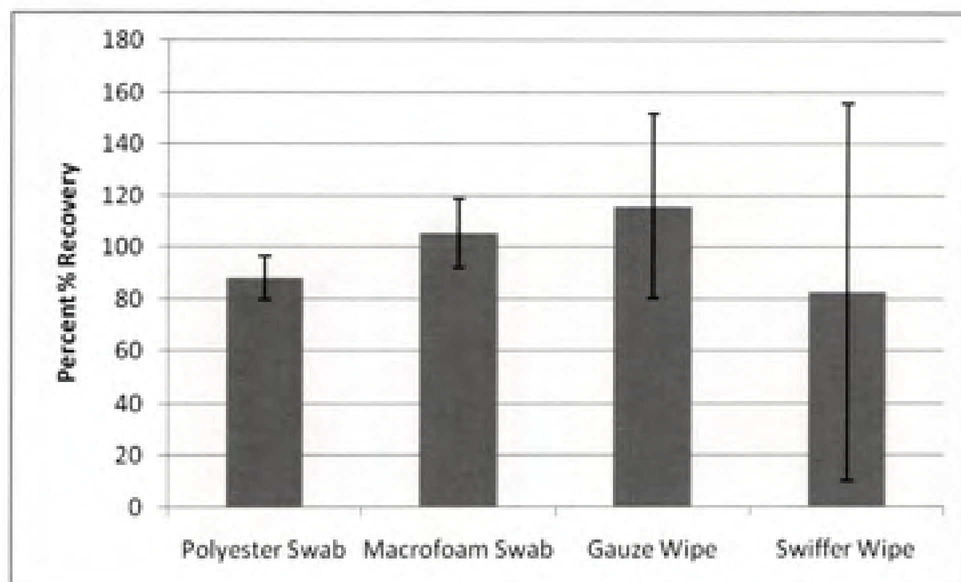

Figure 8: Recovery of Bacterial Inoculum from directly inoculated sampling devices (dry) (n=3, Error Bars are 95% Confidence Intervals)

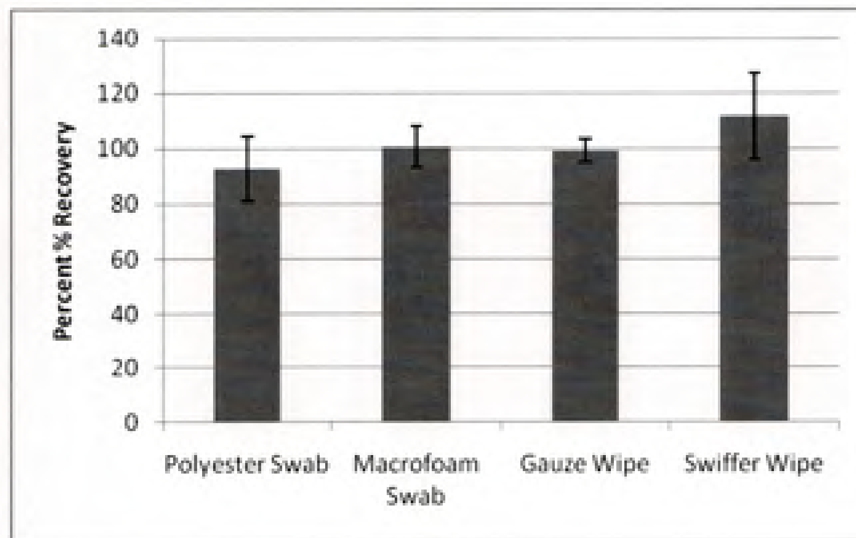

Figure 9: Percent Recovery of Bacterial Inoculate from directly inoculated sampling devices (pre-moistened with DPBS w/ 0.1% Tween 80) (n=3, Error Bars are 95% Confidence Intervals)

6. Sampling Device Evaluation using Ideal Surface

Incorporating what was learned from previous experiments, further evaluation of sampling devices continued to show wide variability in recovery efficiencies between devices and between trials for the same device. Mean recoveries for each sampling device type and inoculum concentration are presented in Table 15 below. Mean recoveries across all inoculua levels for each sampling device are also presented. All experimental runs of this analysis exhibited low mean colony counts and recovery efficiencies across all sampling device trials.

Inoculate Levels	2.8 Log Units w/ Blood		3.2 Log units w/ Blood		3.3 Log Units		4.1 Log Units w/ blood		4.2 Log Units		Overall Recovery across Inoculations	
All Data Points	Average Recovery (%) +/- 95% CI		Average Recovery (%) +/- 95% CI		Average Recovery (%) +/- 95% CI		Average Recovery (%) +/- 95% CI		Average Recovery (%) +/- 95% CI		Average Recovery (%) +/- 95% CI	
Polyester Swabs	0.61	0.47	1.03	1.62	0.20	0.10	1.67	0.22	0.02	0.02	0.71	0.58
Macrofoam Swabs	0.67	0.50	1.97	3.65	0.80	0.61	2.14	1.22	0.38	0.56	1.19	0.71
Gauze Wipes	1.61	2.50	8.63	13.29	11.53	11.35	1.18	0.39	0.24	0.07	4.64	4.47
Swiffer™ Wipes	1.39	1.91	7.82	14.36	0.80	0.12	1.53	1.71	0.12	0.09	2.30	2.73
20% Trimmed Means	Average Recovery (%) +/- 95% CI		Average Recovery (%) +/- 95% CI		Average Recovery (%) +/- 95% CI		Average Recovery (%) +/- 95% CI		Average Recovery (%) +/- 95% CI		Average Recovery (%) +/- 95% CI	
Polyester Swabs	0.46	0.48	0.23	0.35	0.03	0.06	1.64	0.22	0.01	0.02	0.48	0.59
Macrofoam Swabs	0.45	0.48	0.08	0.15	0.40	0.71	2.12	1.39	0.04	0.01	0.62	0.75
Gauze Wipes	0.28	0.54	2.57	2.33	5.87	11.16	1.21	0.40	0.23	0.07	2.03	2.05
Swiffer™ Wipes	1.20	2.09	8.03	15.40	0.35	0.20	1.48	1.66	0.12	0.08	2.24	2.88

Table 15: Mean Recovery of sampling devices on stainless steel coupons at various inoculate concentrations (with and without organic loading-sheep's blood) with 95% Confidence Intervals. (n=3)

Trimming 20% of the data points from each end of the data sets eliminated many of the possible outliers within each trial. Effects on the mean recovery and subsequent confidence intervals can be seen in Table 15, Figure 10 and Figure 11. The mean recovery for each sampling device across all inocula levels is presented in Figure 12. No statistically significant differences between sampling devices were observed when analyzed across all inocula levels either with Trimmed or Untrimmed data (Figure 12).

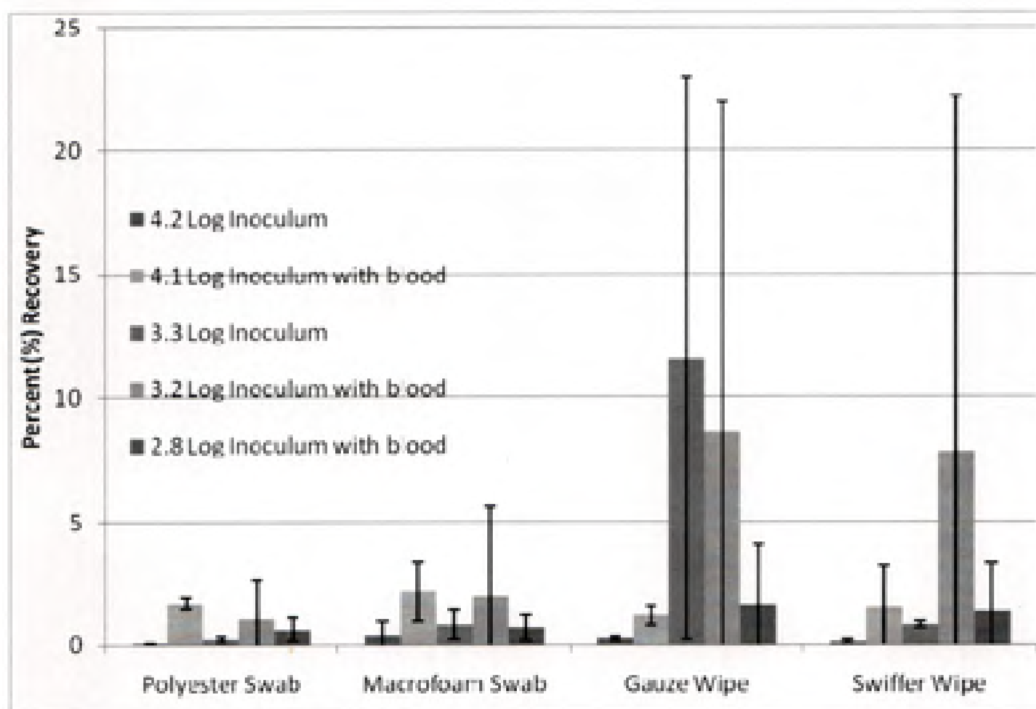

Figure 10: Mean Recovery of sampling devices on stainless steel surfaces (n=3, error bars are 95% Confidence Intervals, Log=Log10).

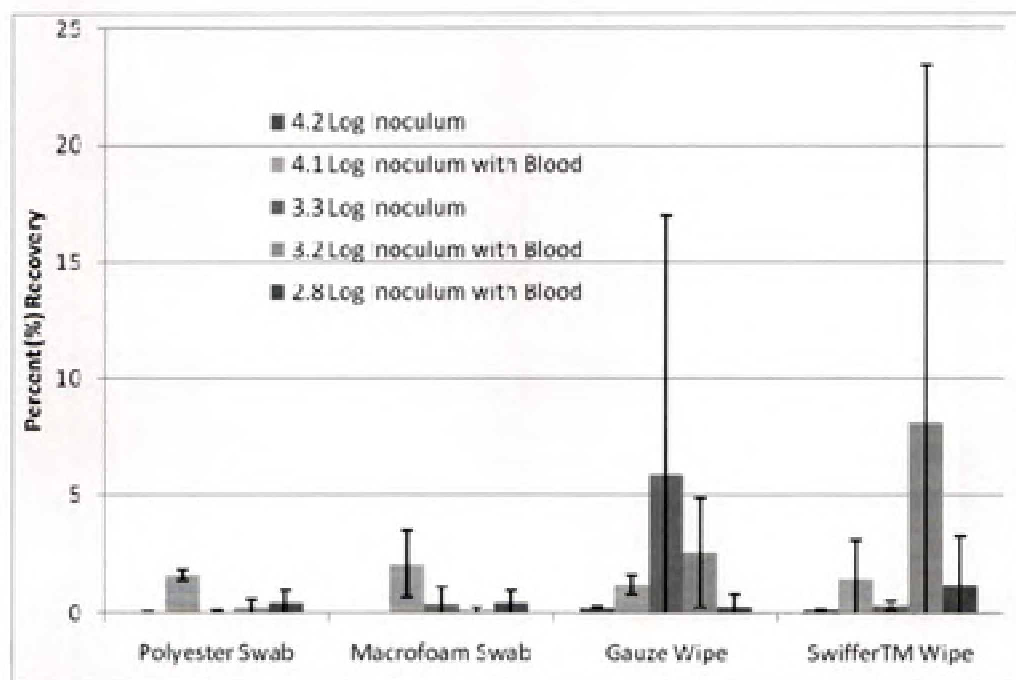

Figure 11: Mean Recovery, based upon 20% Trimmed data, of sampling devices on stainless steel surfaces (n=3, error bars are 95% Confidence Intervals, Log=Log10).

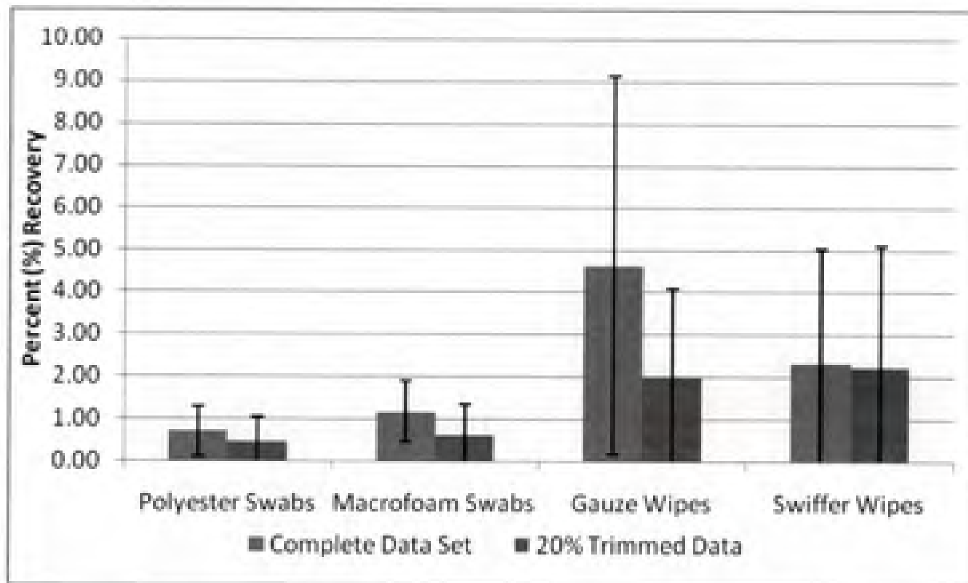

Figure 12: Mean Recovery of Sampling Devices across all inocula levels (n=5, error bars are 95% Confidence Intervals

7. Sampling Device Evaluation using MPN Enrichment Culture to Quantify Test Bacteria

This experiment was conducted under the conditions that the final environmental sampling protocol was to follow. An evaluation of the effects of the enrichment media (TSB amended with 10% NaCl and 1% Na pyruvate) and MPN enumeration method was needed in order to compare candidate methods under these conditions. 3 trials of each sampling device and a high inoculation level (5.6Log_{10}). Table 16 shows the initial test surfaces inoculate concentration and the concentrations of recovered organisms for each sampling device. Overall sampling efficiencies for each sampling device based on an mean inoculum of 5.6 log_{10} are presented in Figure 13. No significant differences were found between sampling devices, although polyester-rayon blend gauze wipes have slightly better recovery. Overall, mean recovery varied between ~0.1 and 1%, with Gauze wipes having the highest mean recovery efficiency. P-values for pairwise

comparisons between the mean recoveries from each sampling device can be seen in

Table 17.

Log_{10} Concentrations of Initially Inoculated and Recovered Organisms (95% Confidence Interval)	
Initial inoculum	5.60
Polyester Swab	2.92 (2.47-3.37)
Macrofoam Swab	2.47 (1.83-3.11)
Gauze Wipe	3.49 (3.00-3.97)
Swiffer™ Wipe	2.39 (1.38-3.39)

Table 16: Concentration of Recovered Organisms (Log_{10} Concentration) based upon 5-tube 3 dilution MPN assay(n=3)

	Polyester Swab	Macrofoam Swab	Gauze Wipe	Swiffer Wipe
Polyester Swab		0.3255	0.1696	0.3948
Macrofoam Swab			0.0688	0.892
Gauze Wipe				0.1252
Swiffer Wipe				

Table 17: P values for pairwise comparisons between sampling devices for mean recovery of test inocula using an MPN method to quantify recovered organisms (Unpaired 2-tailed T-Test, n=3)

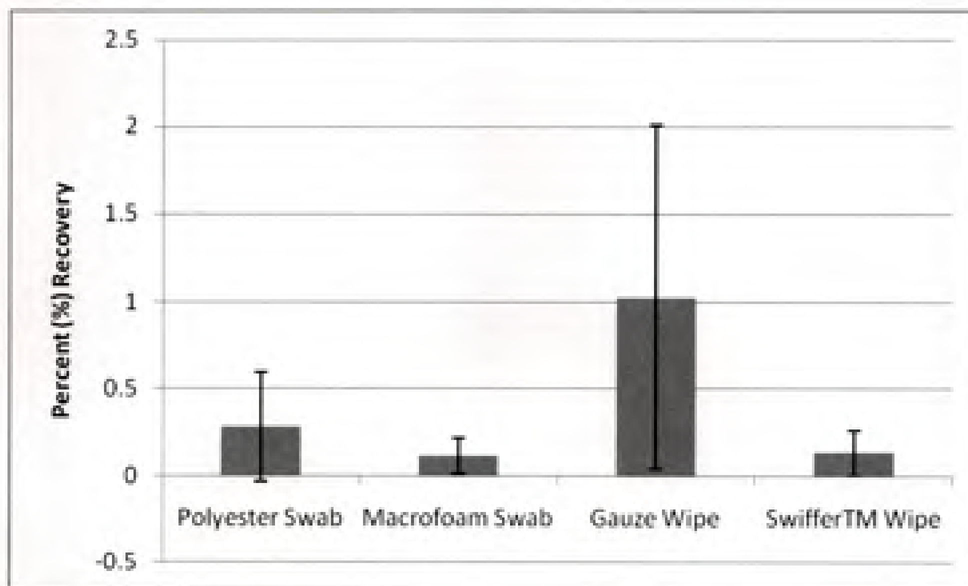

Figure 13: Mean Recovery of Sampling Devices using an MPN Enrichment assay. (n=3, error bars are 95% Confidence Intervals)

C. Phase III-Environmental Sampling

1. Environmental Sampling of Exercise Facilities

Results for S. aureus

The results from Phase III were obtained by sampling the entire surface of a piece of equipment (e.g. the entire bench an exercise or the entire handle section of a dumbbell) using a Polyester-Rayon Blend Gauze wipe as described in "Environmental Sampling of Exercise Facilities" of the Materials and Methods section. Surfaces were wetted with Dulbecco's PBS with 0.1%Tween 80, sampled with a sterile gauze wipe, and placed into 90mL of TSB with 10% NaCl and 1% Na pyruvate in a polypropylene container. All samples were returned to lab and analyzed by a quantal assay (MPN) to estimate the quantity of *S.aureus* present on each surface.

S. aureus was isolated from every facility tested. Positive surfaces as a percentage of total surfaces sampled in the exercise facilities ranged from 19.2% to 72%, with the overall prevalence of *S. aureus* from all samples found to be 42.5% (See Table 18). It should be noted that while Site 2 had the lowest prevalence of *S. aureus*, this site had been cleaned just prior to sampling and no patrons had used the equipment since cleaning. If Site 2 is excluded from the analysis of overall prevalence of *S. aureus* contamination, the rate increased to ~45%.

	No. Positive	No. Negative	% Positive (95% CI)
Site 1	36	14	72.0% (50.4-99.7%)
Site 2	16	43	27.1% (15.5-44.0%)
Site 3	5	21	19.2% (6.2-44.9%)
Site 4	18	23	43.9% (26.0-69.4%)
Site 5	10	6	62.5% (30.0-100%)
Site 6	11	9	55.0% (27.5-98.4%)
Site 7	10	20	33.3% (16.0-61.3%)
Site 8	18	32	36.0% (21.3-56.9%)
Overall	124	168	42.5% (35.3-50.6%)

Table 18: Prevalence of *S. aureus* contamination of surfaces in exercise facilities by site (Confidence Intervals are based on a Poisson distribution)

Surfaces associated with Strength Training represented the majority of surfaces present within the exercise facilities visited and thus made up the majority of samples obtained (n=191). Other exercise types had much lower numbers of samples obtained: Aerobics (n=50), Calisthenics (n=35), and Other surface types (n=16). All sampling sites had equipment designed for Strength Training and Calisthenics, but Sites 3 and 5 lacked any Aerobic exercise equipment, and samples in the Other category (e.g. door handles, locker room samples, etc.) were only obtained from Sites 1, 3, 4, and 6. Ten of the samples in the Other category were from Site 1, while only 2 came from each of the other sites. Prevalence rates based upon exercise category of the sampled surface ranged from a low of 36% for Aerobics to a high of 62.5% for Other (See Table 19).

The highest prevalence of *S. aureus* contamination was found to in the Other category, although the number of samples was small (16) for this category. From Site 1, 3 of 4 sampled door handles were found to be colonized with *S. aureus*. Additionally, 3 of 4 locker room benches from both men's and women's locker rooms were found to have *S. aureus*.

	No. Positive	No. Negative	% Positive (95% CI)
Aerobics	18	32	36.0% (21.3-56.9%)
Calisthenics	20	15	57.1% (34.9-88.3%)
Strength	76	115	39.8% (31.4-49.8%)
Other	10	6	62.5% (30.0-100%)
Overall	124	168	42.5% (35.3-50.6%)

Table 19: Prevalence of *S. aureus* contamination of surfaces in exercise facilities by exercise type (Confidence Intervals are based on a Poisson distribution)

The majority of the equipment sampled in this study either had primary contact with the hands only (e.g. treadmills are only touched directly by the users hands during operation) or had contact with both the torso (usually in the form of a seat or a bench) and the hands (handles). Thus, the majority of samples obtained were associated with hand contact (n=173) rather that torso contact (n=119). The prevalence of *S. aureus* contamination on surfaces associated with the hands was 37.6%, while the prevalence for torso associated surfaces was 42.5%.

	No. Positive	No. Negative	% Positive (95% CI)
Hands	65	108	37.6% (29.0-47.9%)
Torso	59	60	49.6% (37.7-64.0%)
Overall	124	168	42.5% (35.3-50.6%)

Table 20: Prevalence of *S. aureus* contamination of surfaces in exercise facilities by body contact site (Confidence Intervals are based on a Poisson distribution)

A box-and-whisker representation of the quantitative results for *S. aureus* per surface (results are analyzed per whole surface tested) from each category is presented in Figure 14. Median counts from positive surfaces ranged from 0.43 $\log_{10}$ at site 7 to 1.52 $\log_{10}$ at site 2. The overall median concentration per surface was 0.90 $\log_{10}$ (See Table 21

for distribution data for all sample categories). No significant correlation was found between the prevalence of *S. aureus* in samples from each site/exercise type/or Body Contact category and the median cfu count per sample from each of these categories (See Figure 15).

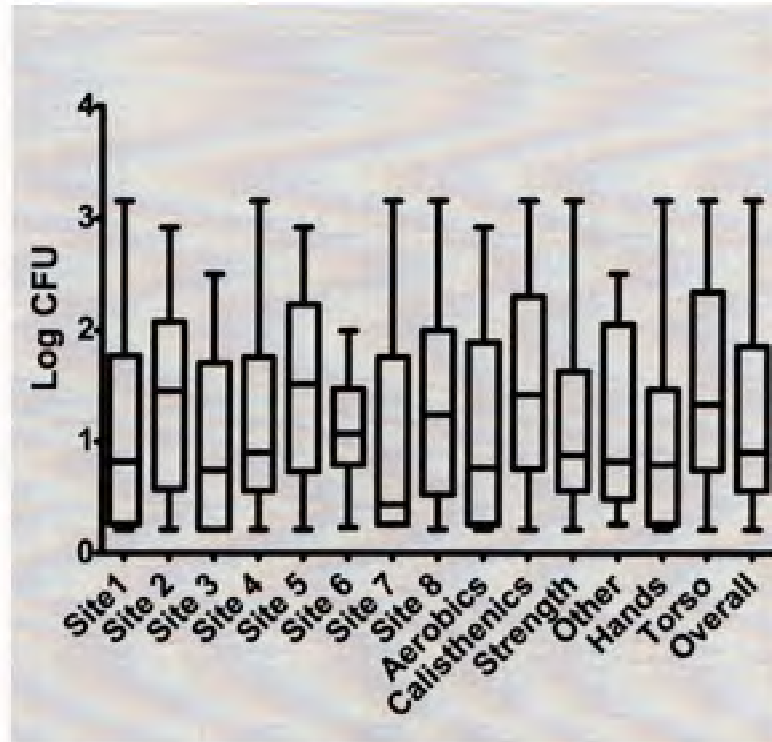

Figure 14: Box and Whisker Plot of the concentrations of *S. aureus* from positive surfaces (Log Transformed). (Whiskers are min to max, results are per entire surface, not a standard area)

Category	Site1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Aerobics	Calisthenics	Strength	Other	Hands	Torso	Overall
Number of values	36	16	5	18	10	11	10	18	18	20	76	10	65	59	124
Minimum	0.21	0.21	0.21	0.21	0.21	0.23	0.26	0.21	0.21	0.21	0.21	0.26	0.21	0.21	0.21
25% Percentile	0.26	0.57	0.21	0.56	0.73	0.79	0.26	0.52	0.26	0.75	0.56	0.48	0.26	0.74	0.56
Median	**0.82**	**1.46**	**0.74**	**0.90**	**1.52**	**1.07**	**0.43**	**1.24**	**0.76**	**1.42**	**0.87**	**0.82**	**0.79**	**1.33**	**0.90**
75% Percentile	1.78	2.07	1.71	1.76	2.23	1.47	1.76	2.00	1.89	2.30	1.63	2.05	1.47	2.33	1.85
Maximum	3.16	2.92	2.50	3.16	2.92	2.00	3.16	3.16	2.92	3.16	3.16	2.50	3.16	3.16	3.16

Table 21: Distribution of log transformed data for the concentrations of *S. aureus* from positive samples from each category of environmental samples

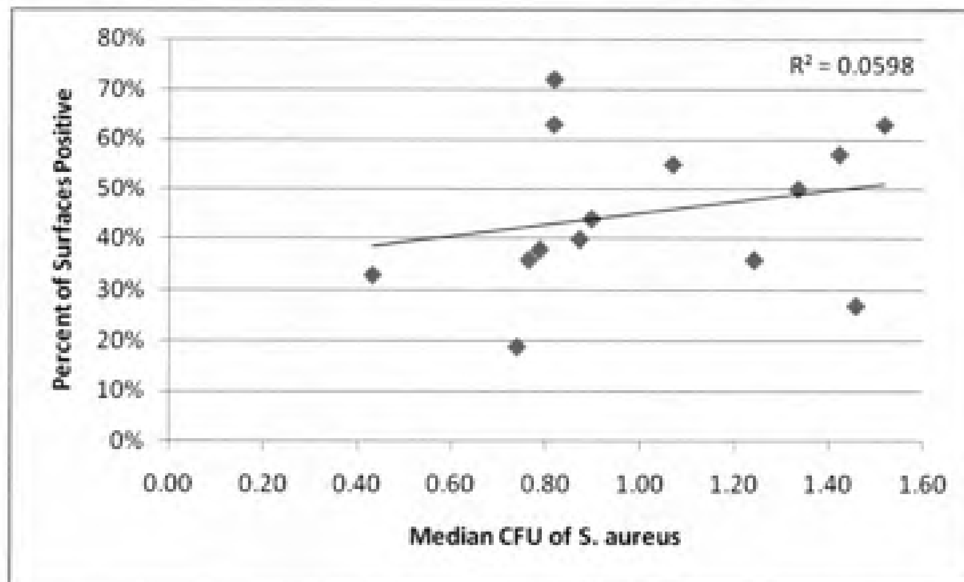

Figure 15: Correlation between Median CFU (Log Units) of *S. aureus* per sampling category and the Percent of Positive Samples in each category

P-values for each pair-wise comparison of *S. aureus* counts on surfaces by type of surface (Aerobic, Calisthenics, Strength and Other) and body contact with the surface (Hands or Torso) are shown in Table 22 and Table 23. There were no significant differences between the median counts for *S. aureus* in only positive samples in each category either between sampling sites (Table 20) or between types of exercise equipment (Table 21), based upon a Mann-Whitney U test. However, the median counts for positive samples obtained from surfaces associated with Torso contact were found to be significantly different than the median counts for surfaces associated with contact with the Hands (P = 0.0026). However, as no attempt was made to standardize this analysis based upon surface area, this finding may be directly linked to the large difference in the mean surface area a Torso contact surface (e.g. a bench) has compared to a Hand contact surface (e.g. a handle). (Table 21)

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8
Site 1		0.3107	0.4354	0.4238	0.1999	0.724	0.4762	0.5255
Site 2			0.3207	0.7034	0.7716	0.3875	0.2538	0.7039
Site 3				0.3304	0.1968	0.3072	0.7078	0.3889
Site 4					0.3999	0.9641	0.1625	0.9117
Site 5						0.3068	0.1696	0.4566
Site 6							0.3185	0.7188
Site 7								0.4124
Site 8								

Table 22: P-values for pair-wise comparisons between the median cfu levels from positive samples from each exercise facility (values are from a Mann-Whitney U test)

	Aerobics	Calisthenics	Strength	Other	Hands
Aerobics		0.1204	0.4732	0.6473	
Calisthenics			0.1642	0.3322	
Strength				0.9408	
Torso					0.0026
Hands					

Table 23: P-values for pair-wise comparisons between the median cfu levels from positive samples from each category of samples (values are from a Mann-Whitney U test)

Results for MRSA

A total of 13 environmental samples out of 292 (4.5%) were found to harbor MRSA with these methods. Sites 1, 2, 4, 7 and 8 were found to have MRSA contaminating at least 1 environmental surface, which is 5 of 8 or 62.5% of all sites. The number of samples positive at each site and in each sample category is presented in Table 24. The highest rates of MRSA prevalence was seen in Site 8 (10%). Site 1, which was also a student exercise facility like Site 8, had a prevalence of 6%. Sites 7 (6.7%) and 4 (4.9%) were personal training facilities with a wide age range of clients, including high

school and collegiate sports teams participants. The highest prevalence of MRSA based upon exercise type was seen in the Calisthenics group (11.4%). Surfaces associated primarily with Torso contact had a higher prevalence (6.7%) than those associated with the Hands (2.9%). Descriptions of the samples positive for MRSA are presented in Table 26.

Category	Pos. for MRSA	Total Samples	% Positive (95%CL)
Site 1	3	50	6.0% (1.2-17.5%)
Site 2	1	59	1.7% (0-9.4%)
Site 3	0	26	0.0% (0-14.2%)
Site 4	2	41	4.9% (0.6-17.6%)
Site 5	0	16	0.0% (0-23.1%)
Site 6	0	20	0.0% (0-18.4%)
Site 7	2	30	6.7% (0.8-24.1%)
Site 8	5	50	10.0% (3.2-23.3%)
Aerobics	3	50	6.0% (1.2-17.5%)
Calisthenics	4	35	11.4% (3.1-29.3%)
Strength	5	191	2.6% (0.9-6.1%)
Other	1	16	6.3% (0.2-34.8%)
Hands	5	173	2.9% (0.9-6.7%)
Torso	8	119	6.7% (2.9-13.2%)
Overall	13	292	4.5% (2.4-7.6%)

Table 24: Number of samples positive for the presence of MRSA and the relative prevalence of MRSA on environmental surfaces (Confidence Intervals based on Poisson distribution)

A comparison of the number of samples positive for MRSA of all samples positive for *S. aureus* is shown in Table 25. The overall rate of MRSA isolation of those samples positive for *S. aureus* was 10.5%.

Category	Pos. for MRSA	All S. aureus	% of S. aureus that was MRSA
Site 1	3	36	8.3%
Site 2	1	16	6.3%
Site 3	0	5	0.0%
Site 4	2	18	11.1%
Site 5	0	10	0.0%
Site 6	0	11	0.0%
Site 7	2	10	20.0%
Site 8	5	18	27.8%
Aerobics	3	18	16.7%
Calisthenics	4	20	20.0%
Strength	5	76	6.6%
Other	1	10	10.0%
Hands	5	65	7.7%
Torso	8	59	13.6%
Overall	13	124	10.5%

Table 25: Percent of Samples that were MRSA out of samples positive for *S. aureus*

Table 26 shows a description of each sample which yielded a MRSA isolate as well as the PFGE strain typing of that isolate. Site 1 had 3 different strains, although 2 of the strains are "Probably related" according to the PFGE analysis. "Probably related" means that the isolates shared all of the same banding patterns on a Western Blot of the digested genetic material of each isolate except for 1-3 bands (Refer to the PFGE Procedure in the Appendix). The isolates from Site 2 and site 4 were also "probably related." These two sites were approximately 30 miles apart, in different counties, and were unlikely to share any patronage. Site7 had 3 distinct isolates, all unrelated to any strains recovered elsewhere. Two of these distinct isolates originated from the same sample. Site 8 had 3 strain types, one of which was an exact match by this analysis to a strain type found at Site 1. Sites 1 and Site 8 are about 30 miles apart and are both Large

University Student Exercise Facilities. Shared patronage other than type of patron is not expected between these 2 sites on a large scale.

Site	Equipment/Surface	Body Contact	Exercise Type	PFGE Analysis
1	Free-weight (30lb)	Hands	Strength	Strain A
1	Exercise/Stretching Mat	Torso	Calisthenics	Strain A_1
1	Women's Locker Room Bench	Torso	Other	Strain B
2	Exercise/Stretching Mat	Torso	Calisthenics	Strain C
4	Exercise Ball	Torso	Calisthenics	Strain C_1
4	Medicine Ball (10lb)	Hands	Strength	Strain C_1
7	Barbell	Torso	Strength	Strain D
7	Treadmill	Hands	Aerobics	Strains E and F
8	Stationary Bike (Seat)	Torso	Aerobics	Strain G
8	Treadmill	Hands	Aerobics	Strain A
8	Exercise/Stretching Mat	Torso	Calisthenics	Strain H
8	Butterfly Press Machine*	Hands	Strength	Strain A
8	Butterfly Press Machine*	Torso	Strength	Strain A

Table 26: Descriptions of Samples that were found to be positive for MRSA. Strain typing by PFGE analysis indicates similarity between isolates from different samples. Strain types with a subscripted numeral are "Probably related" to the isolate with the same letter.
*These samples are from the same piece of equipment

A comparison between the median Log_{10} transformed counts for all *S. aureus* positive samples from each site and category of environmental samples and the prevalence of MRSA is presented in Figure 16. A comparison between the prevalence of all *S. aureus* and MRSA among all groups of samples is presented in Figure 17. There was no observed correlation between the prevalence of MRSA and either median *S. aureus* counts or prevalence of *S. aureus*. Figure 18 shows the distribution of $\log_{10}$ transformed counts in *S. aureus*-positive samples that were later confirmed as MRSA and those found to be MSSA (non-MRSA). There was no significant difference between the

median cfu counts of MRSA and only MSSA positive samples, based on a Mann-Whitney U test (p=0.2731).

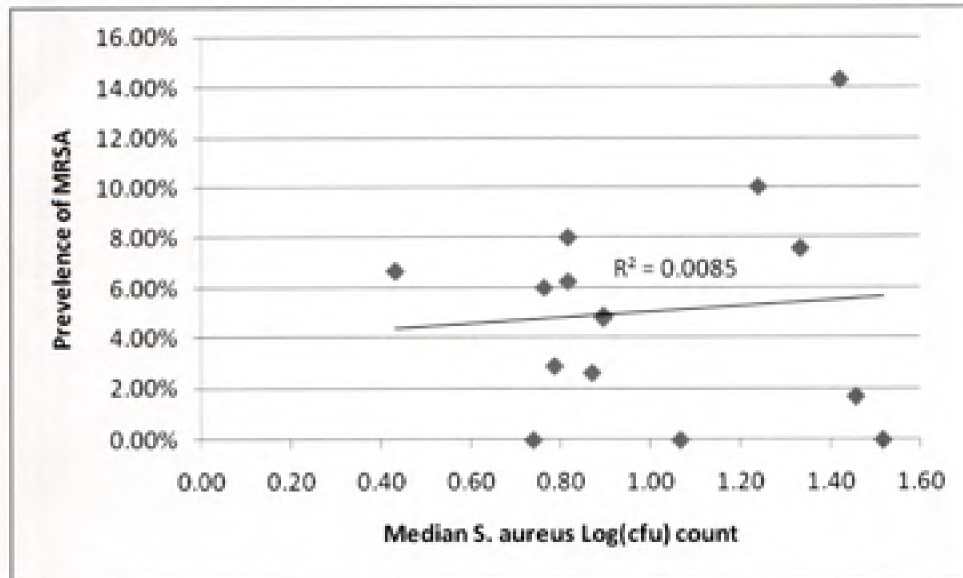

Figure 16: Correlation between Median Log transformed cfu counts for all samples positive for *S. aureus* per category and prevalence of MRSA from each category

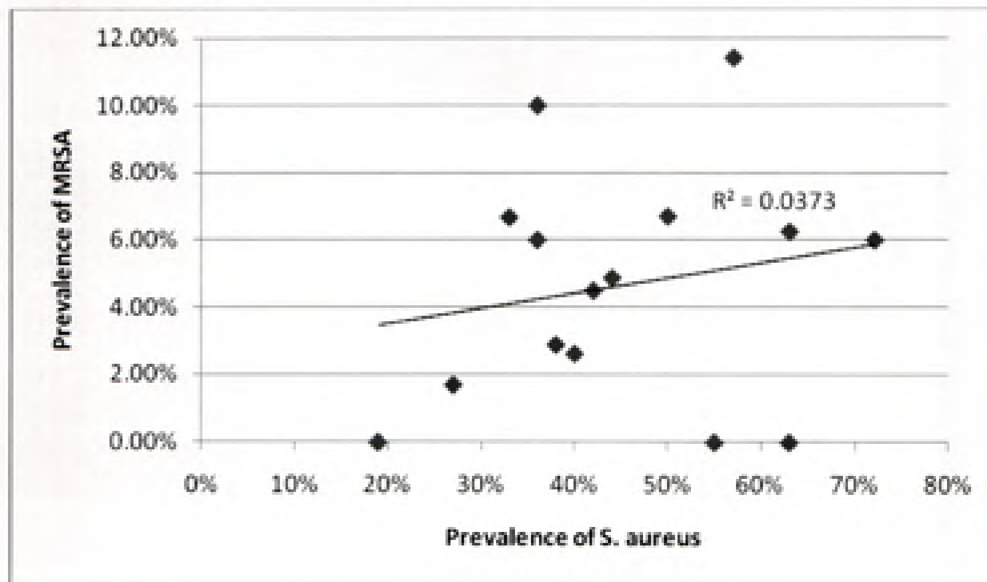

Figure 17: Correlation between the Prevalence of all S. aureus and MRSA from each category

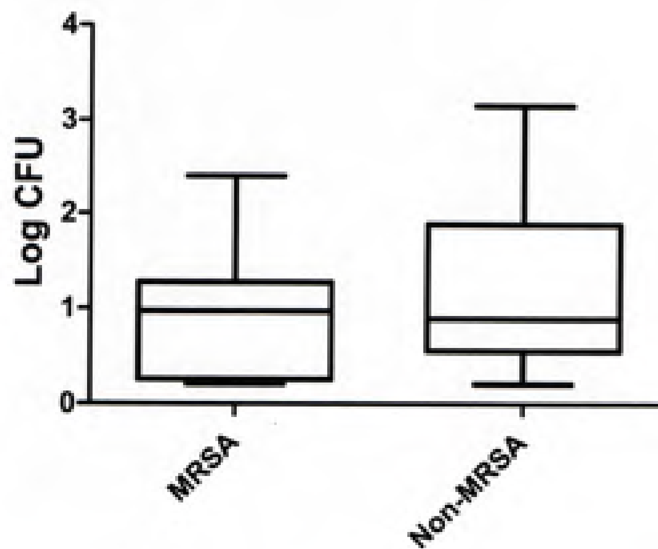

Figure 18: Box-and-Whisker Plot of cfu counts in *S. aureus*-positive samples that were found to be either positive or negative for MRSA (Whiskers are Min to Max)

2. Time-Series Analysis of *S. aureus*/MRSA loading on Gym Equipment

The variability of MRSA/MSSA contamination on environmental surfaces in exercise facilities with repeated sampling over time was investigated. In Table 25 is summarized the equipment included in this experiment, the time points at which these equipment pieces were positive for *S. aureus* and MRSA, and the $\log_{10}$ transformed cfu counts from each positive sample. In Figure 21 is shown the distribution of *S. aureus* and MRSA cfu data from each sampling time point. *S. aureus* was detected at each time point, with 2 to 7 samples out of 10 positive. MRSA was detected on equipment surfaces on all 3 days of sampling. Only once was the same piece of equipment positive for MRSA at both morning and afternoon sampling time points, which occurred on a barbell

bench on day 2. In Figure 19 is a summary of *S. aureus* and MRSA prevalence for each time point, and in Figure 20 is a summary of these data grouped per morning/afternoon sampling times for all 3 sampling days combined and combined morning and afternoon data combined for each of the 3 sampling days. The median value from each comparison of days or between all morning and afternoon samples were found to not be statistically significant by a Mann-Whitney U test.

	Day 1		Day 2		Day 3	
Sample Description	**Morning**	**Afternoon**	**Morning**	**Afternoon**	**Morning**	**Afternoon**
Barbell Bench	-	-	1.352	1.545	3.158	0.732
Barbell Bench	-	-	-	-	-	-
Barbell Bench	0.210	1.276	0.210	-	-	2.276
Barbell Bench	-	2.498	-	-	0.210	0.740
Barbell Bench	-	0.868	2.918	0.210	0.913	1.316
Arm Curl Machine	-	2.068	1.316	0.210	1.879	-
Triceps Press Machine	-	-	0.607	-	-	1.297
Butterfly Press Machine	0.210	-	3.158	1.276	2.334	3.158
Leg Extension Machine	-	-	0.210	-	-	-
Leg Curl Machine	-	0.556	-	0.210	-	0.846

Table 27: List of Samples from Time-Series Analysis Experiment (numbers are Log_{10} CFU's of S. aureus detected. Red Cells are Samples that were found to be positive for MRSA)

Table 28 shows the PFGE strain type of each MRSA isolate from this experiment. Strains isolated across days varied. One cluster of isolates from Day 2 were similar to each other (Strain H).

	Day 1		Day 2		Day 3	
Sample Description	**Morning**	**Afternoon**	**Morning**	**Afternoon**	**Morning**	**Afternoon**
Barbell Bench	-	-	Strain J	Strain H	-	-
Barbell Bench	-	-	-	-	-	-
Barbell Bench	-	Strain F	-	-	-	-
Barbell Bench	-	-	-	-	-	-
Barbell Bench	-	Strain I	-	-	-	-
Arm Curl Machine	-	-	-	-	Strain E	-
Triceps Press Machine	-	-	Strain H_H	-	-	-
Butterfly Press Machine	-	-	-	Strain H_1	-	-
Leg Extension Machine	-	-	-	-	-	-
Leg Curl Machine	-	-	-	Strain H_1	-	-

Table 28: PFGE analysis of MRSA isolates obtained during the TIme Series Analysis Experiment. Strains with subscripted numerals are "probably related" to the strain with the same letter. Strains with subscripted letters are only "possibly related" (i.e. have 3 to 6 bands different from the strain of the same letter)

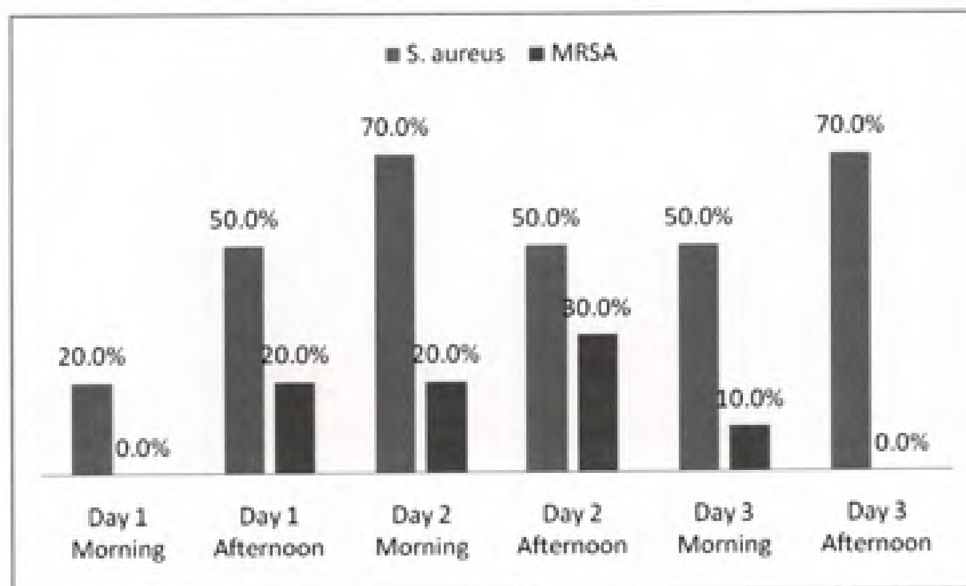

Figure 19: Prevalence of *S. aureus* and MRSA on 10 surfaces in an exercise facility over time

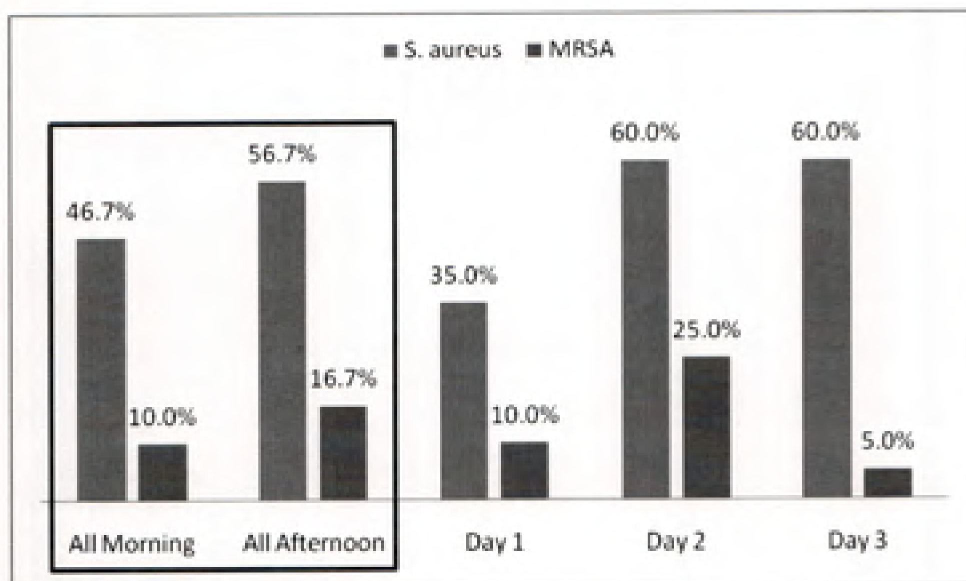

Figure 20: Prevalence of *S. aureus* and MRSA on surfaces from Time-Series Analysis experiment grouped by Sampling Time or Day

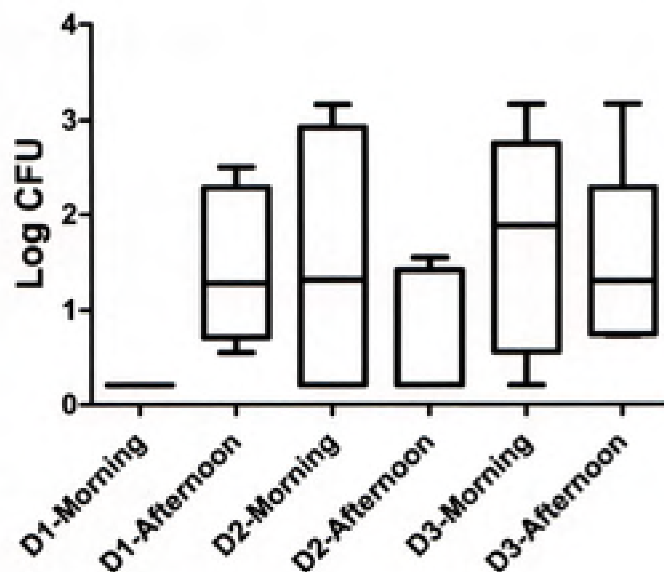

Figure 21: Box and Whisker plot of distribution of colony forming units of *S. aureus* from the Time-Series Analysis at each sampling time point (whiskers are min to max)

IX. Discussion

A. Overview

In this study, *S. aureus* and MRSA were isolated from environmental surfaces which are frequently in contact with peoples' hands and skin in recreational exercise facilities and thus may provide a means of transmission of these pathogens between individuals. These results are novel because there are few studies in the literature that report isolation of these pathogens from this type of environment. Goldhammer *et al.* 2006 attempted to recover these organisms from two exercise facilities on a military base but did not recovery any *S. aureus* or MRSA (29). Of the 8 exercise facilities included in this study, all had frequencies of *S. aureus* surface contamination ranging from 19.2 to72% and 5 had low MRSA prevalence on sampled surfaces ranging from 1.7-10%. The prevalence of MRSA for all study samples was 4.5%. These results are similar to those of healthcare settings, for which reported prevalence of MRSA on surfaces ranged from 0% to 64% with great variation among facilities. The percentage of surfaces positive for MRSA in this community study is higher than found in many previous community studies. In previous studies of community settings, four of six found no MRSA on the following surfaces: various surfaces at a large urban university (9), equipment in gyms on a military base (31), hand railings in public transportation vehicles (72) and telephones at a high school (85). Community setting studies which have reported positive MRSA findings found a prevalence of 8.3% on computer keyboards in university computer labs (41), and 1.34% on household surfaces collected from 35 family domiciles (67).

Isolation of MRSA from equipment and other surfaces inside these public recreational exercise facilities in the absence of any reported MRSA-related disease outbreaks suggests a need for effective measures to protect against transmission by shared use of equipment within these facilities. Recommended control measures include use of proven cleaning/disinfection protocols within these facilities and the promotion of appropriate personal hygiene practices by users of these facilities. Furthermore, it is likely that the contamination rates observed in this and similar studies are under-estimates of the actual rates of MRSA prevalence and concentrations due to the low recovery efficiency of currently available sampling methods.

B. Phase 1

The results from initial sampling of exercise facility surfaces showed that other microbial flora potentially capable of interfering with sampling for *S. aureus* were widely present on environmental surfaces to be sampled. The presence of these other microorganisms indicates that *S. aureus*/MRSA sampling and recovery methods need to not only enhance the recovery of these target bacteria but also suppress the majority of background microbes present. Of the relatively small number (n=10) of samples were taken during this investigation, other *Staphylococcus* species, as well as, *Streptococcus* species, gram-negative bacilli, including *Proteus*, and Gram-positive bacilli, including *Bacillus* species were recovered from all of these surfaces, making it difficult to recover the target organisms. Only 2 colonies of *S. aureus* were isolated from 2 of the surface samples in this initial analysis.

The presence of a range of different environmental bacteria on environmental surfaces is common and the use of various media to select for *S. aureus* and to

differentiate this organism during culture is routine in clinical and industrial food production settings. Most of the literature on environmental sampling for *S. aureus* and/or MRSA does not address the issue of interference from background bacteria, for example, references 2 and 9, used only minimally selective media for the recovery of this target organism. This may not adequately prevent the growth of other microorganisms commonly found on environmental surfaces, and may interfere with recovery of *S. aureus* and MRSA.

The protocols used in most of the previous studies of environmental recovery of *S. aureus* and MRSA do not use methods to enhance the numbers of target organism or selectively recover *S. aureus*. Typical methods used in these studies utilize a swabbing protocol where organisms are put into saline or water and then transferred directly to minimally selective agar media. In this study, a selective medium, TSB with 10% NaCl and 1% Na pyruvate, was used to increase *S. aureus*/MRSA numbers in samples by enrichment culture, and additional selective and differential media specifically designed for the recovery of *S. aureus* were used for subsequent colony isolation. Most other investigations reported in the literature did not incorporate these recovery methods, which makes them less time intensive in terms of sample processing, but probably causes systematic under-estimation of these organisms on environmental surfaces.

C. Phase II

In Phase II, a range of different sampling devices were evaluated in the laboratory using test surfaces representative of those found in exercise facilities as well as idealized (hard, flat, non-porous) surfaces. The investigated sampling devices included Polyester Swabs, Macrofoam Swabs, Polyester-Rayon Blend Gauze Wipes, and Swiffer™ Wipes

(Electrostatic wipes). Sampling efficiencies were low (less than 20%) for all sampling devices and surfaces tested. The mean sampling efficiencies for these devices ranged from ~5-15% for the higher inoculum level on representative surface, ~0.5-5% for all inocula levels on metal coupons, and ~0.1-1% on metal coupons when using a quantal MPN method to evaluate bacterial levels. Gauze wipes displayed the highest mean sampling efficiency in the final evaluation of these sampling devices although replicates were low (n=3) and recovery efficiencies among devices were not significantly different. The high degree of variability observed in recovery efficiencies made it difficult to identify a preferred method. Gauze wipes were chosen for environmental sampling because of they had the practical advantages of ease of use, availability of pre-sterilized units, ability to sample a large variety of surface types without damage, and ability to sample a large surface area quickly.

The high variability and lack of reproducibility for environmental sampling of microorganisms of this study has been previously observed by other investigators. The observed recovery efficiencies in final evaluation of these sampling devices are similar to previously reported efficiencies for swabbing-based methods (50, 52), although the available literature is small. From the results of this investigation, it was concluded that factors such as wetting solution used and excessive retention (inefficient release) of organisms taken up by the sampling device were not major contributors to the overall poor recoveries observed for these types of sampling methods. The wetting/recovery solutions evaluated were not statistically different in performance and ~100% of inoculated organisms applied directly to all sampling devices could be recovered. Similar conclusions were reached by other investigators who analyzed a variety of factors

involved in sampling for *S. aureus* from environmental surfaces and found that choice of sampling device and culture media were the most important factors influencing recovery (52).

The fate of inoculated organisms on the test surfaces was also investigated and approximately 80-90% (n=6 for each of 2 trials) of organisms inoculated onto the test surfaces were immediately unrecoverable. The reasons for this finding were not elucidated may they include cell death, irreversible adsorption to the surface, loss of culturability, or losses in subsequent sample processing steps. Previous studies have demonstrated that *S. aureus*/MRSA can survive from hours to months on common environmental surfaces. However, these studies often used very high initial inocula of the organisms and they do not present quantitative data on organism loss over time (37, 42, 51, 79), which makes it difficult to evaluate the role of bacterial inactivation on surfaces in relation to low recovery efficiency.

Overall results suggest that environmental recovery of *S. aureus* using the investigated methods is low and variable, which may be caused by the various processes involved in the microbial recovery procedure. There is a need to develop new sampling technologies with increase performance in detecting this and other pathogens from environmental surfaces. Our findings suggest that the results obtained for the environmental levels of *S. aureus* and MRSA on environmental surfaces in this and other studies are likely underestimates of the true levels due to the inability of these types of methods to recover these organisms from environmental surfaces.

Our conclusions about sampling efficiencies of these devices and the question about the fate of inoculated organisms on our test surfaces are based upon a fairly limited

investigation of these factors. Including other methods for evaluating the numbers of remaining viable organism like re-sampling each test surface, utilizing additional culture methods like applying agar to each test surface after sampling to enumerate remaining bacteria, or utilizing molecular methods like PCR analysis may have provided additional and more rigorous information about the colony forming unit levels of our test organism and allowed for a better evaluation of sampling efficiency and other factors, like environmental survival, associated with environmental surface sampling.

D. Phase III

In this study the prevalence of surface contamination with *S. aureus* from all facilities ranged from 19.2% to 72%. MRSA prevalence within each site ranged from 0-10% for surfaces sampled, with 5 of 8 facilities having at least one surface positive for MRSA. The overall prevalence of MRSA on all surfaces sampled in all 8 exercise facilities was 4.8% (14 of 292 total samples from all facilities). The type of equipment with the highest prevalence of MRSA was the Calisthenics group (11.4% or 4 of 35 samples). Median colony forming units of *S. aureus* recovered on positive surfaces was $0.90 Log_{10}$. Colony counts across all surfaces displayed a large degree of variability, with colony forming units on some surfaces above the assay detection limits ($\geq 3.16 Log_{10}$ cfus). There were no statistically significant differences between the median colony forming units recovered:

1. between each facility
2. between each group of exercise equipment
3. between surfaces which were positive for MRSA and those negative for MRSA but positive for MSSA.

Surfaces having contact with the users torso had a higher median cfu count ($1.33Log_{10}$) for *S. aureus* than those having contact with the user's hands ($0.79Log_{10}$) (Mann-Whitney U test, p=0.026). This difference in microbe level may be directly linked to Torso contact surfaces having higher surface areas than Hand contact surface areas. In this study the median cfu counts in relation to surface area was not directly examined because surface area per sample was not estimated and/or recorded. Recording the surface area for each sample, or standardizing the sampling area would have made comparisons between all groups of samples easier. This was not done however, as we wanted to include the entire area of each environmental surface to increase our chances of detecting our target organisms and measuring every surface would have limited the number of samples we could obtain even further due to time constraints.

A time-series analysis of MRSA contamination on 10 surfaces suggests that the rates and levels of contamination of these organisms vary and are transient, in that they do not persist over time or change in a predictable manner. Recent contamination by colonized individuals may be the primary driver of contamination, but the specific user-related reasons for differences in microbe occurrence and level were not investigated.

Pulsed-Field Gel Electrophoresis analysis of MRSA isolates obtained during the environmental sampling phase and time-series analysis experiments shows that contaminating strains of MRSA on these types of surfaces are diverse. Some similarities between isolated strain types were found in this analysis, but the reasons for these similarities are unclear. The most striking similarity was observed between some of the isolates found at both Large University Student Exercise facilities. These facilities should not share a large proportion of patrons, but are relatively close to each other and

these Universities may share a small number of students. Finding similar isolates from these sites may indicate that the community of students of college age in this area share common strains of MRSA. An epidemiological analysis of the MRSA strains in the local community may address this question.

This investigation was limited by the relatively small number of exercise facilities that were sampled due to time limitations and difficulties in facility recruitment. Proposed sampling of environmental sources for MRSA in commercial exercise facilities created fears about negative publicity resulting from potential MRSA findings, and hence was a problem encountered in recruitment. Additionally, this study is limited due to the relatively poorly understood effects that the poor sampling efficiency of our sampling device/method imposed. In future studies, the addition of more rigorous sampling methods or alternative testing methods like PCR-based techniques may be useful in constructing a better picture of the actual levels of these pathogens on environmental surfaces.

Previous searches for MRSA in the community setting have yielded mostly negative findings (7, 29, 70, 83); however, one study found 8.3% of computer keyboards inside university computer labs (39) were contaminated with MRSA, and in another study 1.34% of household surfaces were contaminated (26% of all households had at least one positive surface) (66). Prevalence of MRSA contamination found on environmental surfaces from investigations inside hospitals range from 0 to 64% (See Table 6 and Table 7). The only other known investigation conducted in exercise facilities did not discover any *S. aureus*; although, only 2 facility sites were included in this study and only 20 samples were taken during the surveillance phase of this study (29). There

were more samples, 292, and the use of methods to select for and increase the numbers of *S. aureus* and MRSA in this current investigation that were not used in the previous study. These differences may have contributed to the negative findings of the previous study.

Comparisons between this current study and previous community-based studies is challenging because most of the existing literature does not present quantitative data on the number of colony forming units of target organisms found on environmental surfaces, instead presenting only presence/absence data. Investigations conducted in the healthcare environment are more likely to present quantitative data, and be based on a standardized sampling area. One study conducted on patient room door handles found between 1 and 2.6×10^4 cfu of MSSA and up to 6×10^3 cfu of MRSA on these surfaces. Furthermore, the distribution of microbe counts followed roughly the distribution observed in this current investigation (i.e. 38 of 53 cfu counts from patient room door handles were $\leq 1 \text{Log}_{10}$) (54).

The findings of this study suggest that there is prevalent *S. aureus* contamination on environmental surfaces inside recreational exercise facilities. Additionally, the detection of MRSA on surfaces in the majority (5 of 8) of the facilities investigated suggests that there may be some risk of acquiring this pathogen through the use of these facilities. The extent to which such exposure is likely to increase the carriage rates of MRSA in the general population is unknown and deserves further investigation. Because consequences of infection with these pathogens can be serious, it is recommended that exercise facilities have a comprehensive cleaning and disinfection protocol in place to combat the environmental surface contamination risks posed by these pathogens. Users

of these facilities should be instructed in safe personal hygiene habits such as wiping down equipment before and after use and showering prior to and after exercising.

E. Summary and Conclusions

Little is known about the role of environmental transmission of *S. aureus*/MRSA. There is a need to better understand the distribution of these pathogens on environmental surfaces and the risks posed by such environmental exposures. There have been reported cases where the environment has been implicated as a potential vehicle of spread of MRSA in both the healthcare and community settings, but investigations of these outbreaks have often been limited by the inability to find the causative organisms in the environment after the recognition of the outbreak. (5, 9, 11, 16, 17, 20, 36)

Attempts at investigating MRSA in the environment have employed a variety of methods, but have often relied upon swabbing-based methods. Other methods have been developed, including contact-based techniques (e.g. contact/Rodac plates and agar coated dip-slides, etc) which have been shown to be highly efficacious for flat, non-porous surfaces (52, 59). However, these contact sampling methods have limited applicability to the "non-ideal" surfaces which make up the majority of environmental surfaces with which people come into contact. New, more advanced sampling techniques like wash/rinse methods, vacuum sampling, field portable sampling kits and other technologies are being developed and tested, mostly for use in bioterrorism investigations. However, such methods have not been widely adopted as they are difficult to use for repeated or multiple sampling. This is because they are meant to sample a large surface area, but are difficult to use for collecting more than just a few samples at a time

(25). In this investigation, only swabbing based methods were screened for field use because of the limitations in the other sampling methodologies currently available.

From the results of sampling method investigations, it was found that sampling and specimen processing protocols would best be aimed at selecting for and enriching the numbers of target organism while suppressing the other micro-organisms that are present on target surfaces. Many previous investigators of environmental *S. aureus*/MRSA do not report the use of a reliable and validated method for selecting for target microbes and against background flora when sampling for MRSA. Therefore, previous investigations of environmental MRSA may have underestimated MRSA levels on surfaces due to the ineffectiveness of the methods in recovering this organism from samples heavily contaminated with other environmental microorganisms. However, the extent to which this possibility contributed to the reported results is difficult to evaluate for the existing literature.

The evaluation of candidate sampling devices yielded results consistent with those of other researchers based on efficiencies of swabbing-based methods for the recovery of *S. aureus* (~1%) and confirmed that such modifications as choice of wetting agent resulted in only minor differences in the overall low recoveries of these methods (50, 52). However, evaluation of sampling devices was further complicated by the observation that approximately 80-90% of the inoculated bacteria were unrecoverable for poorly understood reasons. Our evaluation of microbe survival may suggest that recovered organisms are only those that 1) survived on the surface, 2) were not lost during the subsequent processing steps of these procedures, and/or 3) represent the net effects of real or apparent loss resulting from adsorption to the surface or clumping of cells on number

of available colony forming units available for recovery. The recovery efficiencies of ~0.1-1% from the sampling device evaluations of this study may be closer to 1-10% if 90% of the inoculum applied to the surface looses culturability. However, this possibility needs further exploration under controlled laboratory conditions. If observed losses are occurring due to adsorption/die-off processes, it is possible that the recoverable fraction of the total population of organisms on environmental surfaces represents the hardiest subset of initially deposited organisms not strongly adsorbed to these surfaces. More investigation into these questions will need to be done if we are to better understand the role of environmental surfaces in the occurrence and spread of pathogens like MRSA.

Based on the results of initial sampling and recovery studies, the observed prevalence and levels of target bacteria obtained during environmental sampling are likely to be lower than their actual prevalence rates and levels of contamination present on the surfaces in these facilities. However, perhaps the field findings represent only those organisms readily transferable to users of these pieces of equipment, and thus may be appropriate estimates of occurrence and levels for use in future quantitative risk assessments of MRSA transmission from surfaces. Many of the remaining unanswered questions about sampling efficiency need to be addressed in order to fully understand what the findings of this and of many other environmental investigations mean in terms of pathogen exposures and their risks.

Despite the shortcomings in current sampling methods and knowledge of the distribution and fate of these pathogens on environmental surfaces, the findings of this study show that *S. aureus* and MRSA are present on user contacted surfaces inside recreational exercise facilities. These findings lend support for the implementation of

regular and effective cleaning protocols in these types of facilities (for which there is no current required standard), and the need to educate the public about the use of hygienic practices by patrons of these facilities. Furthermore, additional work is recommended to evaluate the risk posed by environmental surfaces in the transmission of these pathogens, especially MRSA, whose carriage rates in the population continue to increase with time.

X. Recommendations for Future Work

- Investigate and attempt to develop better method s for the recovery of these pathogens from the environment, especially from environmental surfaces
- Elucidate the fate of *S. aureus* and MRSA on various types of environmental surfaces and their partitioning between a bound and unrecoverable and relatively unbound and transferrable state
- Develop methods and data for a quantitative risk assessment model for this organism from exposure to environmental surfaces
- Continue further environmental sampling in order to investigate other variables like effects of patronage magnitude and type, cleaning agents/protocols, conditions related to hygienic practices and other exposure-related variables on the levels of MSSA/MRSA on these surfaces.

XI. Appendix I-Form Letter Sent for Recruitment of Exercise Facilities

Greetings,

My name is Joseph Hatch and I am a graduate student at the UNC School of Public Health. For my research project, I am working to gather data on the prevalence and levels of Methicillin-resistant *Staphylococcus Aureus* (MRSA) on various pieces of equipment and other surfaces inside public recreational and sports facilities. *S. aureus* is a bacterial pathogen of humans that can cause a wide variety of infections ranging from cutaneous/skin infections to respiratory tract infections to systemic infections in the blood stream and other body sites. The most common type of *S. aureus* infection is the cutaneous type and these infections most often resemble rashes, boils, or other types of non-healing open wounds. This organism is carried by about 25% of the population, often residing deep in the nasal cavity without causing any problems; infections occur when the organism is transferred to a susceptible site like a skin abrasion or cut.

MRSA is *S. aureus* as well, but it has acquired certain traits that have made it more resistant to methicillin, which is a second generation penicillin-based drug. Additionally, certain strains of MRSA have acquired additional resistance factors against many of the other types of antibiotics commonly used to treat bacterial infections. It is speculated that MRSA strains first developed in hospitals, but over the years MRSA has also been isolated from normal, healthy individuals outside the hospital setting, and the spread of Community Associated MRSA (CA-MRSA) has become of great concern.

My study will involve taking samples from various surfaces from exercise equipment inside various facilities and testing them for the presence of both routine *S. aureus* and MRSA as well as the collection of some demographic information of the patrons and the cleaning protocols followed at each facility. The information that I collect from each facility will remain confidential, with no names being used during the reporting phase of my work. Each facility will be privy to the results from their own facility and if the owner/manager of each facility so chooses, we may be able to address any major concerns that may arise once the results of the study are known.

This project is a joint venture between the UNC School of Public Health Environmental Sciences and Engineering Department and the Department of Epidemiology at UNC Hospitals. My advisors are Dr. Mark Sobsey (UNC-SPH), Dr. David Weber and Dr. Bill Rutala (UNC Hospitals).

If you would be amenable to allowing me to include your facility in my study, or if you have further questions about the study, please contact me via email at hatchjo@email.unc.edu; otherwise, I will be calling each potential facility over the next couple of weeks in order to address any questions or concerns as well as to potentially set up a time that may be convenient for me to visit your site. The sampling part of this research should begin sometime in mid to late fall and should be wrapping up by the end of the year (2009).

Thank you for your time and I look forward to hearing from you,

Joseph Hatch

XII. Appendix 2-Pulsed-Field Gel Electrophoresis Protocol at UNC-Hospitals for MRSA strain typing

Pulsed-Field Gel Electrophoresis Protocol
(Reviewed 3/24/09)

1.) Prior to making your isolate blocks used in the PFGE procedure, the organisms are plated to Brain Heart Infusion Agar, Trypticase Soy Agar, or Sheep Blood Agar. However, when working with *Serratia marcescens* or *Neisseria gonorrhoeae*, your isolates must be no older than 18 hrs. (Note: When time is a problem, isolates, with the exception of *S. marcescens* and *N. gonorrhoeae*, can be subbed and kept at room temperature 3-5 days.) For optimum results, it's best if all subs are no older than 18hrs.

For *Staphylococcus sp.* and all Gram-negative rods (GNR's) (See below for *S. marcescens* and *Enterococcus sp.* Protocols):

1.) **(For GNR's)** Make your EC Lysis buffer – lysozyme solution immediately prior to use by mixing 0.01 gms (10 mgs) of lysozyme (in lab freezer) with 10 mls EC lysis buffer. This gives us a concentration of 1 mg/ml lysozyme. Let this solution sit at room temperature until the lysozyme has dissolved. You can expedite this process by incubating this solution at 37°C.

2.) Now's a good time to make your 2% agarose by taking .2 gms agarose and mixing it with 10 mls TEN. (Note: In order to make enough 2% agarose for just 15 blocks, use 50 mg of Incert low melting point agarose and 2.5 ml TEN.) This smaller volume is best made by placing the low melting point agarose in a small sterile tube, adding the TEN, and heating it gently over a low bunsen burner flame. Take care not to let the agarose bubble out of the tube onto your hand. Also be careful not to heat it so much that a significant amount of evaporation occurs and changes the concentration of your solution. Heat the mixture until the agarose is almost dissolved, then place the tube in a 55°C block incubator so it stays in the liquid form.

3.) Now you're ready to begin making your plugs. The following are the suggested volumes dependent on the organisms you're pulsing:

a.) GNR's – [5 colonies of organism (1/2 inch swipe) + 30 ul 0.9% sterile saline + 20 ul TEN + 100 ul EC Lysis Buffer – lysozyme solution] + 150 ul (equal part to "[ ]") 2% agarose

b.) *C. difficile* – [25 ul of 10x organism inoculum + 100 ul lysozyme + 25 ul TEN] + 150 ul 2% agarose

c.) *Staphylococcus aureus* / CNSS / *Neisseria gonorrhoeae* – [5 colonies of organism (1/2 inch swipe) + 30 ul 0.9% saline + 120 ul TEN + 0.5 ul lysostaphin (0.6 ul for CNSS)] + 150 ul 2% agarose

These combinations can be placed in a sterile eppendorf tube or a small sterile test tube. Take care to place all contents in the bottom of the tube.

4.) Once all the components are combined, quickly place them in your plug mold which has been cleaned prior to this point by soaking in the bleach bath, rinsing with distilled water, and dried.

5.) While allowing the plugs to harden, prepare the wells in the tissue culture plate by:

a.) **(For GNR's)** ...placing 1 ml of EC Lysis – lysozyme solution in each well.
b.) **(For *Staphylococcus aureus* and CNSS)**...placing 1 ml of EC Lysis solution (without lysozyme) with 1.25 ul of lysostaphin (for Staphylococcus aureus) or 6.25 ul of lysostaphin (for CNSS).

6.) After the plugs have hardened, place one plug in each well. Allow plugs to rotate gently for 1 hr. at room temperature.

7.) Clean plug mold with Vesphene, rinse with water, and allow to air dry. Immediately prior to the next use, soak mold for 30 min in bleach bath, rinse with distilled water, and let dry. Mold is ready for use.

8.) After rotating your plugs for 1 hr., prepare your ESP solution by adding 100 ug of Proteinase K per 1 ml ES buffer (i.e. 0.1 mg/ml) for each isolate (So, if you have 14 isolates, you will add 1,400 ug or 1.4 mg Proteinase K to 14 mls ES buffer). (Note: Proteinase K is located in lab freezer) Once the Proteinas K has dissolved, withdraw EC Lysis solution currently on the plugs and replace it with 1 ml ESP solution.

9.) Incubate plugs in ESP solution overnight at 37°C.

10.)Some good stalling points are:

- Your plugs can sit in Proteinase K upto 4 days at room temperature.
- After you perform your three rinses, following incubation in ESP solution, your plugs are safe to sit at 4°C until you are ready for their evaluation.
- After the rinse following digestion, your plugs are safe to sit at 4°C.

11.)After overnight incubation in ESP, withdraw ESP solution and rinse plugs with 1 ml of TE(LC= Low Concentration) and allow them to rotate 1 hr. at room temperature. After 1 hr., withdraw old TE(LC) and add fresh TE(LC) and allow it to rotate another hour. After this hour, rinse one more time with TE(LC). After this hour, withdraw old TE(LC) and add 1 ml of fresh TE(LC), and keep them at 4°C until you're ready to cut them.

12.)Now your plugs are ready to be cut. Prior to cutting, label the 96 well tissue culture plate and fill the wells with 300 ul of DNS (Dummy No Salt) solution. This rinses away the salts that could interfere with the enzyme digestion.

13.)Cut your blocks on the lid of a petri dish with the help of a ruler. First, excess fluid is removed from the plug by touching its corner with a piece of Kimwipe. Then, trim the very edge of the plug with a sharp scalpel in order to make it straight. Then, a 5 mm piece is measured from the straight edge and cut. This piece is then cut in half (or a little less). We now have a 5 mm x 4 mm slice of plug. (Note: In order to manipulate the blocks, bend the end of a straight media spatula to a 90° angle. This will be used to lift the blocks out of their wells. You will also need to bend a needle in the same manner to use in order to retrieve the blocks from the 96 well plate.) After all the plugs have been cut, and the appropriate pieces have been placed in their respective wells, the DNS is withdrawn, and 300 ul of fresh DNS is added to each well.

14.)Then, cover the plate with a sheet of parafilm and allow it to rotate at room temperature for 1 hr.

15.)While this plate is rocking, pull the restriction enzyme buffer out to thaw. Prepare enough buffer so you have 100 ul of buffer for each step. There are two steps, one rinse

with just buffer and one rinse with buffer and enzyme. This buffer solution can only be made 1 hr. prior to use. It is made by combining 300 ul of Neb buffer + 30 ul BSA + 2670 ul sterile water = 3000 ul. If you only have a few blocks to pulse, cut these volumes in half.

16.)After your plugs have rotated 1 hr. in DNS, withdraw the DNS and add 100 ul of your buffer solution to each block. Rotate the plugs in this solution for 1 hr. at room temperature. Prior to rotating, confirm that each block is lying down appropriately and is covered by the buffer solution.

17.)After rotating 1 hr., prepare your enzyme-buffer solution by adding 1 ul of the appropriate enzyme for each 100 ul of buffer needed (100 ul / block). Withdraw the old buffer solution and add 100 ul of the new enzyme-buffer solution to each isolate. Allow them to rotate at room temperature overnight. Once again, make sure each block is covered by the enzyme-buffer solution.

18.)After the plugs have digested overnight, draw off the enzyme-buffer solution and add 300 ul TE(HC) buffer. Allow plugs to rotate at room temperature for 1 hr.

19.)While the plugs are rotating, make your gel and prepare the electrophoresis chamber:

- The chamber is prepared by mixing 900 mls 1xTBE with 900 mls RO polished water, which gives us a concentration of .5xTBE. Once in the chamber, set the chiller to cool this buffer to 14°C.

- The 1% gel is made by mixing 1.6 gm of agarose (BioRad Chromosomal Grade Agarose) with 160 mls of .5xTBE made previously (80 mls 1xTBE + 80 mls RO polished water). Heat the agarose mixture in the microwave briefly, until the agarose dissolves. Be careful not to let the agarose boil and overflow out of the flask. Once the agarose is dissolved, place the flask in the 55°C water bath in the CPA and let the mixture equilibrate and cool.

20.)After your plugs have completed rotating in the TE(HC) buffer, position them on the migrating edge of the comb, facing the body of the gel. (Note: Use the needle bent in a 90° angle to manipulate the plugs from the 96 well tissue culture plate to the comb.) Once all the plugs have been placed on the comb, use a Kimwipe to draw off the excess fluid. This keeps them from sliding off the comb when positioned vertically. It also aids in keeping all the bands aligned during the electrophoresis process. Finally, position the plugs evenly on the bottom edge of the comb. (Note: Always place the lambda ladder in your first well as your quality control. Some people choose to place the lambda ladder in the last well in addition.)

21.)After making sure the mold is centered, position the comb in the mold and make sure the comb is pushed all the way to the back of the slot. Then, gently pour the agarose in the mold, at the back of the plate so the fluid doesn't force the blocks to come off the comb.

22.)Allow the gel to harden 15 – 30 min.

23.)After the gel has hardened, gently lift the comb out of the agarose and dismantle the mold. Remove the gel plate and position it in the chamber. Close the lid and begin BioRad Chef. Run the gel at switch times of 5 secs. / 35 secs. For 23 hrs. at 200 volts (Exceptions for these switch times are recorded). Be careful not to reach in the chamber while the current is running.

24.)After 23 hrs., the gel is ready to be stained and photographed.

25.)Place gel in ethidium bromide stain in HLA photography room. Allow it to sit in the stain 30 min to 1 hour.

26.)Photograph gel by placing it on the camara stage. Focus the camara by viewing the wells. Once the wells are in focus, place on your safety glasses (located by camara) and turn on the black light. Increase the exposure times until a small amount of red appears on the screen. Adjust the contrast to 100% and decrease the brightness as far as you can without losing any bands. Finally, print out your photograph.

For *Enterococcus sp.* and *Serratia marcescens:*

27.)Place a small, fresh inoculum of each *Enterococcus sp.* isolate in a separate, sterile, conical tube containing 10 mls TSB and allow to incubate at 37°C for 5 hrs.

28.)After 5 hrs, centrifuge tubes for 10 min and drain off supernatant.

29.)Once all the supernatant has been removed, add 1 ml TEN to each pellet and vortex on each tube on high for 1 min.

30.)Make blocks by combining 150ul of each suspension to 150ul agarose, pipet into block mold, and allow to solidify.

31.)Once the blocks have solidified, place each block in a well containing 1 ml EC Lysis Buffer and lysozyme. Then, allow them to incubate at 37°C overnight.

32.)After incubating overnight, remove the EC Lysis buffer and lysozyme from the blocks using a transfer pipet and add 1 ml ESP sol'n. Allow this to incubate at 56°C for 5 hrs.

33.)After 5 hrs., draw off the ESP sol'n and rinse the blocks with TE(LC) buffer for 1 hr. at room temperature. After 1 hr., remove the old buffer and add fresh TE(LC) and allow to shake 1 more hr. at room temperature. Finally, after 1 hr., rinse one more time with fresh TE(LC) buffer. After this hour is up, add fresh TE(LC) buffer, cover the bottom portion of the microtiter plate with parafilm, place the lid of the plate on and refrigerate. At this point, you're ready to cut your blocks with their specific restriction enzyme when time allows.

34.)To continue, refer to steps 12) – 26).

General PFGE Reagents:

(Note: Calculation example for EDTA: 1M = 372.2 (Molecular Wt.) gm/L
1mM = .3722 gm/L
100 mM = 37.22)

1.) Lysostaphin (Sigma: L-0761 – 5mg Protein)
- Prepare stock at 10mg/ml in 20 mM Na Acetate (0.16406gm/100ml)
(So reconstitute our vial with 500ul Na Acetate)
- Store at 4°C.

2.) Lysozyme (Sigma: L-6876 – 10gm)
- Prepare at 1mg/ml in EC Lysis Buffer immediately prior to use. It is not stable in alkaline solutions.

3.) **Proteinase K (Sigma: P-2308 – 100mg)**
- Prepare at 100ug/ml in ES Buffer/isolate immediately prior to use.

4.) **TBE (1x)**
89 mM Tris (216gm/20L or 5.39gm/500ml)
89 mM Boric Acid (110gm/20L or 2.75gm/500ml)
2 mM EDTA-Na dihydrate (14.88gm/20L or 0.37gm/500ml)
- No pH adjustment

5.) **TE (LC-"Low Concentration")**
10 mM Tris (1.21gm/L or 0.61 gm/500ml)
1 mM EDTA (0.37gm/L or 0.19 gm/500ml)
- pH to 7.9

6.) **TE (HC-"High Concentration")**
100 mM Tris (12.11gm/L or 6.06 gm/500ml)
100 mM EDTA (37.22gm/L or 18.61gm/500ml)
- pH to 7.9

7.) **TEN**
100 mM Tris (12.11gm/L or 6.06gm/500ml)
100 mM EDTA (37.22gm/L or 18.61gm/500ml)
150 mM NaCl (8.77gm/L or 4.39gm/500ml)
- pH to 7.9

8.) **0.9% Saline**
9 gm NaCl/L

9.) **Dummy No-Salt Buffer (DNS)**
100 mM Tris (12.11gm/L or 6.06gm/500ml)
5 mM $MgCl_2$ (5ml of 1M stock/L or 0.48gm/500ml)
- pH to 8.0

10.) **Ethidium Bromide**
10 mg/ml
- Store at 4°C in a dark bottle

11.) **EC Lysis Buffer**
6 mM Tris (0.73 gm/L or 0.37gm/500ml)
100 mM EDTA-Na2 salt, dihydrate (37.22 gm/L or 18.61gm/500ml)
1M NaCl (58.44 gm/L or 29.22gm/500ml)
0.5% Brij-58 (5 ml/L or 2.5gm/500ml)
0.2% Na Deoxycholate (2 ml/L or 1gm/500ml)
0.5% Na Sarkosyl (N-Lauryl Sarcosine, Na salt) (5 gm/L or 2.5gm/500ml)
- pH to 7.9 (Use NaOH)
- Requires alkaline pH for solubilization
- STIR SLOWLY

12.) **ES Buffer**
500 mM EDTA (186.1 gm/L or 93.05gm/500ml)
1% Sarkosyl (10 gm/L or 5gm/500ml)
-pH to 8.5-9.0 (Use NaOH)
-Requires heating for solubilization

Notes:

- Low Melting Point Agarose – Incert Agarose – Catalog # 50121 ($29.00) – FMC BioProducts, 191 Thomaston St., Rockland, ME 04841 USA

- All Restriction Endonucleases must be stored at –20°C in a **NON**-frost free freezer

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