Differences in the Survival, Transmission, and Susceptibility to Disinfection of M1 and M5 Protein Possessing *Streptococcus pyogenes* and Other Pathogens on Airplane Cabin Surfaces

by

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Abstract

Recent reports regarding the transmission of *Mycobacterium tuberculosis* and other microorganisms in airplane cabins compel us to examine the factors for pathogen transmission in that environment. We tested the survival and transmission of *S. pyogenes* (Group A *Streptococcus* or GAS), the causative agent of diseases such as Strep Throat, Necrotizing Fasciitis, and several other maladies on surfaces taken from airplane cabins. This is a first step in determining risk factors for transmission of bacterial diseases in airline cabins. GAS strains and either PBS or artificial saliva were mounted onto sterile airplane surface coupons and incubated before determining survival under environmental conditions similar to airplane cabins in flight. Pigskin was used as a transmission vehicle to determine transmissibility of *S. pyogenes* from cabin surfaces to skin. Transmission rates were determined by placing GAS and artificial saliva or PBS onto coupon surfaces then uniformly pressing these swatches onto pigskin. GAS possessing the M1 protein survived for a minimum of 18 days, regardless of solution or surface. GAS possessing the M5 protein was inhibited by saliva, surviving a minimum of 6 days. M1 serotypes were transmissible for a minimum of 12 days while M5 serotypes were for 4 days. These findings indicate that this bacterium easily has the ability to be transmitted in airplane cabins by contact/surface transmission. To provide a solution for our results, we also investigated the use of a hypochlorous acid product (Nature Unleashed™ Anolyte) for disinfection

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Preface

My research had 3 goals directed towards understanding the survivability and transmissibility of *S. pyogenes* under airplane cabin conditions in hopes of finding ways to reduce the risk of transmission to passengers in the future.

• Goal 1: Determine Survivability Using Airplane Cabin Coupons

S. pyogenes strains with M1 and M5 M protein types were applied to coupons to determine the survivability of the organisms in different substances on each surface under typical airplane conditions.

• Goal 2: Determine Transmissibility Using Airplane Cabin Coupons

S. pyogenes strains with M1 and M5 M protein types were applied to coupons then directly touched with pigskin to determine transmissibility of the organism from the surfaces.

• Goal 3: Disinfection of Airplane Cabin Coupons

A hypochlorous acid derivative disinfectant was applied to *S. pyogenes, Escherichia coli* O157:H7, and Methicillin Resistant *Staphylococcus aureus* mounted on surface coupons to determine effective disinfection methods that might be used in airplane cabins while unoccupied on the ground.

Chapter 1

Literature Review

1. Airplane Travel and Disease

Approximately 3.3 billion passengers were transported via aircraft in 2014 [1]. These passengers represent almost every established country, including areas with a higher prevalence of certain transmittable diseases [2]. Airplane travel contributes frequently to the spread of emerging diseases that were once confined. For example, the transport of Ebola virus in 2014 from a localized area in Africa to the United States occurred via airplane transport of infected persons[3]. Exhaustive screening of all airline passengers and cargo for the presence of every communicable disease is not currently a viable option, so possible transmission aboard airplanes remains an area of great concern to public health and safety. In order to address the potential risks for outbreaks of infectious organisms aboard aircrafts, it is critical to develop an understanding of the survivability, transmissibility, and appropriate routes of disinfection for pathogens under specific aircraft conditions.

2. Disease Transmission on an Aircraft

The risk of infectious disease spread on an airplane can change due to different conditions on board and the duration of the flight. Airplane layouts are typically designed so that many passengers sit in a restricted amount of space, increasing the risk of disease transmission due to the high occupant density. Long flight times can even allow passengers to be exposed to some pathogens and toxins for the entirety of their incubation periods [4]. Further, airplane cabins have a low relative humidity of about 14-19% [5]. Organisms such as *Staphylococcus* as well as viruses are more viable as airborne particles in lower relative humidities [6].

To address these problems, high-efficiency particulate air (HEPA) filters that capture 99.9% of particles 0.1-0.3μm in diameter are installed in most newer model airplanes [7].

However, according to Pall Corporation, a global supplier of filtration, separations, and purification products, "There are currently no airworthiness standards or regulations which specify the level of filtration removal efficiency which must be used on board aircraft." The majority of modern, large, commercial aircraft, which use a recirculation type of cabin air system, utilize fine HEPA filtration, (99.99% minimum sodium flame efficiency/99.97& minimum DOP efficiency). A small number of aircraft types have filters with lower efficiencies. Some older aircrafts have either total outside air ventilation, or a small amount of unfiltered recirculation combined with the outside air." [7]. Regulations in place for airplanes are uncertain, from HEPA filter placement to the cabin cleaning schedules of individual airline companies. The presence of potential carriers of disease and immunocompromised individuals also increases the risk. Due to the large volume of people who fly on airplanes, prolonged identification time for particular diseases, and limited resources for testing in some geographic locations, it is not feasible to assess or test each passenger for the presence of infectious disease before their flight. All of these risk factors have contributed to many past instances of pathogen transmission and outbreaks on board airplanes.

3. Transmission Instances on an Aircraft

Documented outbreaks that occurred during air travel involve *Mycobacterium tuberculosis*, Influenza, Severe Acute Respiratory Syndrome (SARS), Meningococcal Disease, and the Norovirus [8]. These instances of transmission could have occurred through contact or airborne transmission. *M. tuberculosis*, the causative agent of TB, is responsible for the largest recorded number of airplane outbreaks and is an ideal organism for spread by airborne transmission [8].

3.1 Tuberculosis

Tuberculosis (TB) is one of the most common infectious diseases in the world [9, 10]. In fact, almost one-third of the world's population are infected with TB; an estimated 8.6 million people became infected in 2012 and around 1.3 million people died from the disease. TB is transmitted through airborne contact initiated by the coughing, speaking, or sneezing of infected people. Once internalized, the bacterium typically infects the lungs and can present through the symptoms of cough, chest pain, weakness or fatigue, weight loss, coughing up blood (hemoptysis) and/or sputum, night sweats, loss of appetite, chills, and fever. HIV positive people are particularly at risk for the disease; in 2012, 320,000 HIV positive people died from TB. Treatment for *M. tuberculosis* is antibiotic therapy, however, drug resistant strains have developed and present a heightened public health risk [9,10].

There is evidence that *M. tuberculosis* can be transmitted on airplanes [10]. The risk of contracting TB while on board an airplane relates to the duration of exposure to the person with the disease, the infectiousness of the person with TB, proximity to the infected person, and whether proper ventilation is utilized in the airplane cabin [10]. The baseline information for the risks of TB transmission on an airplane was largely gathered from two instances of transmission, one in 1992 and one in 1994 [11]. The 1994 transmission incident involves a 32-year-old woman who flew on 2 flights, then a month later, 2 more flights. She had been treated as an adolescent for TB, and again within the past two years. In between the two sets of flights, the index patient's symptoms progressed from coughing to lethargy, fever, and hemoptysis. A week after her final flight, the index patient died and was diagnosed with multi drug resistant TB. Health departments and the CDC obtained flight records then test results and demographic information from people exposed to the index patient. With this information, they were able to determine that sitting within 2 rows of the infected person increases the risk of TB transmission by 8.5 times [11].

The second instance of transmission was in 1994, and involved a flight attendant who had a previous positive skin test conversion but was not treated for TB [11]. While working as a flight attendant, she developed a severe cough and was diagnosed with TB. Upon analysis of the skin test results and demographic data from flight crews and passengers, investigators concluded that those exposed to the index patient for longer than 12 hours were at a higher risk for transmission of the pathogen. In addition, primarily only other flight crew members had positive skin test and conversions, indicating that the duration of exposure, proximity to infected person, and repeat exposures to the index patient increases risk. The conclusion from these two instances was that TB could be transmitted aboard airplanes. Further, the World Health Organization (WHO) began publishing *Tuberculosis and Air Travel: Guidelines for Prevention and Control*, which is still in publication today [11].

3.2 SARS

Severe Acute Respiratory Syndrome (SARS) is caused by a coronavirus and was first discovered in 2003 [12]. SARS causes a form of pneumonia with a death rate or 12% in younger people but over 50% in older people [13,14]. The virus is transmitted through droplet spread by coughing or sneezing. Additionally, the virus can survive in a droplet on fomite surfaces for up to 6 hours. Like *S. pyogenes*, SARS can be spread by contact and surface transmission in addition to airborne transmission

Discovered only 12 years ago, the spread of the virus to new areas of the world was tied to airplane travel. During the initial years of discovery, many outbreaks of SARS on board airplanes were documented [13,14]. Specifically, 9 of the first probable SARS case patients traveled on a commercial aircraft for 8 flights total [14]. One of these flights, a 3-hour flight in March, 2003, carried a symptomatic 72-year-old passenger who was later diagnosed with SARS.

When contacted during investigations after the incidence of transmission, 23 passengers on the flight reported to have contracted SARS and 5 of these people died from their illnesses. All infected persons reported an onset of the illness approximately four days after the flight, the typical incubation period for the virus. Most of the infected people were also seated within 3 rows of the index patient and none identified previous or other instances of potential exposure to SARS. The conclusion of the SARS outbreak was that, like TB, the instance of transmission heavily weighed on proximity to the infected patient. In addition to airborne routes of transmission, contact and surface transmission were suggested as a possibility [14].

3.3 *N. meningitidis*

Most bacterial meningitis cases in the United States are caused by *Neisseria meningitidis* [15]. *N. meningitidis* is a Gram-negative diplococcus that is spread from person-to-person by direct contact [15]. Prolonged close contact heightens the risk of exposure, and the attack rate among people sharing a household with an infected person is 500-800 times greater than the general population [16]. In humans, *N. meningitidis* causes an infection of the meninges, or the lining surrounding the brain and spinal cord [17]. If untreated, meningococcal meningitis caused by *N. meningitidis* is fatal in 50% of cases. Symptoms of infection include, high fever, confusion, light sensitivity, a stiff neck, and vomiting. Early diagnosis is crucial, around 10% of patients die within 24-48 hours of infection. Due to the direct contact spread of this organism, transmission aboard an airplane has been documented and remains a risk. Those seated directly next to an infected person for longer flights are at an escalated risk. However, the widespread vaccination campaigns in the United States in 2010 significantly lessened the instance of *N. meningitidis* infection and, consequently, the risk of in-flight spread [17].

3.4 Norovirus

Noroviruses are single stranded RNA viruses that cause gastroenteritis [18]. There are 3 genotypes of the virus that infect humans, and all 3 are highly contagious. Symptoms can develop within 12 hours post exposure and last 24-72 hours. Symptoms include vomiting, diarrhea, abdominal cramps, low-grade fever, headaches, and body aches. Dehydration is a very common result of a norovirus infection and can be serious in the elderly and very young.

Norovirus infections are the leading cause of gastroenteritis in the United States with 19-21 million illnesses a year. With a virus occurring this commonly, there have been numerous documented outbreaks among airplanes [18]. On October 1, 2009, a passenger became sick on a Boeing airplane, vomiting while in flight [3, 19]. Between the dates of October 13-18, 43% of the flight attendants working on the airplane met the definition of a norovirus case. A unique property of the norovirus is that it must be ingested to cause infection. For this reason, it is likely that the flight attendants were exposed to the virus by touching a contaminated surface then ingesting the virus. Further, though exposure to the virus did decrease in the days after the index patient's flight, some attendants were still infected. This supports the possibility that transmission of appropriate pathogens can occur on airplanes by contact and fomite transmission [19]. The fact that attendants were still infected days after the vomiting instance proves that a more intensive cleaning regime is necessary to prevent prolonged transmission of some pathogens.

4. *Streptococcus pyogenes* Overview

Streptococus pyogenes, a member of family *Streptococcaceae* and exclusive Lancefield Group A pathogen, is a Gram-positive coccus arranged in chains [20]. The organism is a strict human pathogen that causes around 750 million infections and over 650,000 deaths per year, making it one of the world's major human pathogens [21, 22, 23]. *S. pyogenes* colonizes the

mouth, throat, upper respiratory tract, and vaginal tract [24]. Typically, *S. pyogenes* causes common illnesses such as scarlet fever, strep throat, and impetigo [24, 25]. However, the organism can also cause extensively invasive group A streptococcal disease, including streptococcal toxic shock syndrome and necrotizing fasciitis.

5. History of *S. pyogenes*

S. pyogenes possesses a remarkable history of disease variation, dating back to the 1700s [24, 25]. In the 18th and 19th centuries, the organism was responsible for many deaths of women during or around the time of childbirth due to puerperal fever. Since GAS can colonize the vaginal tract, healthcare workers or the natural process of childbirth would expose the bacteria to the uterus, causing a systemic and often fatal infection. Since the introduction of hand washing practices, puerperal fever has become largely uncommon worldwide. During World War II, *S. pyogenes* was responsible for streptococcal gangrene or necrotizing fasciitis infections of soldiers. These infections caused more deaths than actual war related deaths. Necrotizing fasciitis largely "disappeared" until it resurfaced in the 1980s and 1990s, causing a, "the bacterium that ate my face" response from the media.

Also around the time of World War II, scarlet fever emerged. Primarily a childhood disease caused by *S. pyogenes*, Scarlet fever was characterized by a diffuse red rash that was remarkably contagious and transmissible by airborne and foodborne contact. As with necrotizing fasciitis, scarlet fever cases were almost nonexistent or considered a mild childhood disease between the years of 1940 and 1990, when the disease resurfaced. Upon the reemergence of scarlet fever, the disease was much milder but still caused outbreaks among children in close contact with each other.

After the 1940s, GAS was linked to glomerulonephritis, a skin infection that led to inflammation of the kidneys, as well as rheumatic fever in adults and children [24,25]. Rheumatic fever develops from an untreated case of GAS pharyngeal infection and can spread to heart tissue and valves, causing severe irreversible damage to the heart and possible heart failure [20]. Rheumatic fever mysteriously disappeared from developed countries and has not resurged, possibly due to the use of antibiotics [24,25]. However, glomerulonephritis is still common today, indicating that antibiotics disproportionally affect different strains. In addition to the resurgence of scarlet fever and necrotizing fasciitis, a *S. pyogenes* toxic shock-like syndrome and a swiftly fatal pneumonia have recently become concerns.

Though strep throat caused by *S. pyogenes* remains the most common disease of the organism, the repertoire of other more serious illnesses also caused by GAS are prominent. Typically, *S. pyogenes* is extremely sensitive to Penicillin and it is the most common treatment regime for GAS pharyngitis infections, however, some strains have evolved resistance to the antimicrobial. With its history of disease variation and pathogenic nature, *S. pyogenes* is a pathogen that should not be overlooked [24,25].

6. Clinical Characteristics

In a clinical setting, *S. pyogenes* is notable for exhibition of β hemolysis, a clear zone of complete lysis on blood agar produced by Streptolysins S and O [20,24,25]. Many *Streptococcal* species exhibit this hemolysis, which inspired Rebecca Lancefield to develop a classification scheme to further differentiate β hemolytic *Streptococcus* species based on polysaccharide antigens in the cell wall. Lancefield groups are classified in groups A-E, with *S. pyogenes* belonging to Group A *Streptococcus* or GAS. *S. pyogenes* is classified as a Biosafety Level II pathogen and no vaccine is currently available [20, 24, 25].

7. Virulence Factors

Serotyping of GAS is based on the antiphagocytic M protein, also called "M type" or "*emm* type" [21]. There are over 100 distinct M types, of which immunity is specific to each type [21, 26]. However, few M types are linked to the majority of invasive infections. M1 and M3 protein possessing GAS isolates have been linked to the largest proportion of infections, including necrotizing fasciitis and toxic shock syndrome, rendering possession of M1 or M3 proteins particularly notable [21,26]. Though M1 protein strains cause multiple infections, many strains of GAS are classified as exclusively skin or throat infection causing serotypes [23]. However, some organisms that colonize the skin have the ability to colonize the throat as well.

Both the M proteins and GAS lipotechoic acid (LTA) are located on fimbriae, outside of the cell wall. Both are virulence factors of *S. pyogenes*, with the M protein helping the organism resist phagocytosis and LTA mediating attachment. This attachment to human and extracellular matrix cells is the beginning of *S. pyogenes* infections. Attachment is critical. Without strong adherence, *S. pyogenes* cells could be washed away by mucous or saliva [23]. The lipid moiety of the lipotechoic acid (LTA) is primarily responsible for *S. pyogenes* attachment, interacting with fibronectin and epithelial cells of the host [27]. In addition to LTA, the M protein, other proteins, and the hyaluronate capsule have all been linked to attachment [27].

7.2.1 Survival in Saliva

In the oropharynx, GAS is exposed to human saliva which has a great many components, including water, histatins (polypeptides with anti-bacterial activity), proline-rich proteins (PRP), mucins, lysozyme, secretory immunoglobulin A (sIgA), crystatins, blood plasma components, and other various mucosal secretions and proteins [28, 29, 30]. Understanding how GAS responds to saliva is necessary since the organism is constantly exposed to the bodily fluid

during pharyngeal infection. Further, *S. pyogenes* must survive in saliva in order to be transmitted to a new host through respiratory droplets [29].

S. pyogenes growth in saliva consists of an exponential phase and a prolonged stationary phase [29]. During early interactions with human saliva, GAS was shown to have high transcripts of genes involved in oxidative stress, such as *aphC* (encodes peroxiredoxin reductase), *mtsA* (encodes a metal binding protein), and genes in the arginine deaminase operon *spy1541-8* [31, 32]. During 4-16 hours of growth in saliva, studies show that GAS have increased transcripts in genes encoding proteins involved in the metabolism of carbon sources such as Amy A and MalX (starch degrading proteins), AgaD and MalE (phosphotransferase enzymes), Sic, SpeB, and carbohydrate metabolism enzymes. Further, strains possessing the M1 protein persist longer in saliva than strains with other *emm* types [31].

Possession of the M1 protein, *sic* and *speB* genes, and the *sptR/S* two component regulatory system contributes significantly to the ability of GAS to survive and proliferate in saliva. The M1 protein is the most prominent *emm* type in causing *S. pyogenes* infections today. This is likely due to the possession of the *mga* regulon, illustrated in **Figure 1**. This is a regulon composed of gene *mga* followed by *emm1*, *sph*, and *sic.* Gene *sic* encodes for Sic protein, which is responsible for evasion of complement-mediated lysis of erythrocytes and inactivation of antimicrobial peptides such as lysozyme. The extreme sensitivity of *S. pyogenes* to penicillin provides an interesting explanation for Sic's ability to inactivate lysozyme. GAS is hypothesized to inactivate the antimicrobial peptide in order to allow lysozyme sensitive beta-lactamase producers to survive in the oropharynx. These organisms release beta-lactamase in addition to protecting themselves from beta lactam antibiotics, affording *S. pyogenes* a safeguard against penicillin. This could in part explain the ability of the organism to colonize the oropharynx for

extended amounts of time, even after appropriate antibiotic treatment. The survivability, transmissibility, and disinfection of GAS possessing the M1 protein versus GAS possessing a non-M1 protein is a cornerstone of this study [31].

7.1 M protein

The M protein of GAS is located on the surface of the organism and ultimately functions to inhibit phagocytosis of the organism by the host immune response [33, 34]. The protein is a dimer of two chains composed of α helix coils that is anchored in the cell wall peptidoglycan via an LPxTG motif and sortases. The attachment within the cell wall occurs by a transpeptidation reaction that cleaves a surface protein substrate at the LPxTG cell wall sorting signal.

All 80+ types of M proteins share a hypervariable amino terminus, central domain, C-terminus, and signal peptide as shown in **Figure 2**. Following the hypervariable region are repeat sequences A-D. Most M proteins do not possess A repeats, however; C repeats are present in all M proteins in varying amounts [33,34].

M proteins bind to proteins such as collagen, fibronectin, blood protein factor H, IgA, IgG, and fibrinogen [24]. In binding IgG, instead of binding to the normal antigen-binding site, M protein binds to the Fc portion. This prevents the antigen-binding sites from binding the surface of the bacteria. The M protein is further antiphagocytic in its binding to complement factor H, which prevents factor H from aiding in the activation of C3b to bind to the bacterial surface and initiate phagocytosis. Lastly, M protein binds to proteins like fibrinogen to function in adhesion [24]. M proteins are reported to bind salivary mucin, which is necessary for GAS colonization of the host [24, 34, 35].

7.2 M1

M1 protein serotypes, as aforementioned, are exceptionally virulent. Responsible for the majority of severe GAS infections since the 1980s as well as a number of common pharyngeal episodes of infection, the protein possesses a regulatory gene called *mga* [24]. Gene *mga* is followed by *emm1* (M1 protein encoding gene) and *sph*. Gene *sph* encodes protein H, which binds to IgG Fc region. Following *sph* is *sic* [24, 26, 36].

7.3 Streptococcal Inhibitor of Complement (Sic)

Gene *sic* codes for an extracellular protein hypothesized to contribute to the ability of M1 GAS to better survive in saliva than other serotypes (4, 16, 25, 41). Sic, the streptococcal inhibitor of complement protein, is a hypervariable protein only secreted by M types 1 and 57. This rare protein functions to inhibit complement mediated lysis of sensitized erythrocytes. Further, Sic inactivates antimicrobial peptides like lysozyme, and α and β defensins [31, 37, 38, 39]. Though Sic is found in only 2 M types, over 300 variants of the protein have been identified [26, 36, 40]. In contrast to the other products of *mga* regulon, Sic does not have a COOH terminus anchored to the cell wall through an LPxTG motif. Instead, it is secreted. Since Sic is missing a cell wall anchor, it is typically found in growth medium [26, 36, 40].

7.3.1 Sic and Lysozyme

Sic is advantageous to GAS isolates possessing the M1 protein due in part to its ability to inhibit the function of lysozyme [36, 40]. Research by Fernie-king et al. (2004) showed that Sic does not have enzymatic activity against lysozyme when incubating Sic and lysozyme together [36, 40]. Sic likely works by blocking the catalytic or antimicrobial site of lysozyme. Since GAS is a Gram-positive organism and typically resistant to lysozyme with cell walls 15-17 nm thick, the inhibition by Sic is particularly interesting. One reason Sic might inhibit lysozyme's function is due to the synergistic bacterial killing effect of lysozyme and other antimicrobial substances

[36, 40]. For example, the interaction of lysozyme and lactoferrin has been shown to kill a variety of bacteria in a capacity that lysozyme or lactoferrin alone cannot replicate [36, 40, 41]. Therefore, if Sic inhibits lysozyme, it could have a better chance of survival in the oropharynx.

7.3.2 β lactamases

In previous studies, tonsils removed from children with recurring GAS adenotonsillitis were colonized with many organisms, including β lactamase producers [35, 42, 43]. This leads to the second possible explanation for Sic activity against lysozyme. Most *S. pyogenes* strains do not produce β lactamase, an enzyme produced by bacterial species that hydrolyze the endocyclic peptide bond (ultimately, the beta lactam ring) in beta-lactam antibiotics [44]. However, due to the heightened sensitivity of GAS to penicillin, a beta lactam antibiotic, creating a symbiotic relationship with β lactamase producing bacteria (BLPB) could allow *S. pyogenes* to avoid penicillin activity [45, 46, 47, 48, 49]. For this reason, it is hypothesized that GAS produces Sic, which inhibits lysozyme, to allow β lactamase producing bacteria (BLPB) that are sensitive to lysozyme to survive in saliva. These BLPB can in turn hydrolyze the beta lactam ring in penicillin, providing protection for GAS against the antibiotic, as shown in **Figure 3**. Many BLPB provide this protection to penicillin susceptible species by releasing free enzyme into the environment. The relationship between BLPB and GAS has been the object of numerous experimental studies and is heavily documented [45-50].

7.4 Streptococcal Pyrogenic Exotoxin, SpeB

Another protein linked to the survival of *S. pyogenes* in saliva is Streptococcal Pyrogenic Exotoxin (SpeB) [51]. The *speB* gene, unlike *sic*, is found in almost every GAS serotype. However, the amount of SpeB protein is variable among the strains of GAS. Strains of *S.*

pyogenes able to persist in saliva for longer than 7 days have been shown to produce a detectable amount of SpeB while strains that cannot survive do not.

SpeB, a cysteine protease with fibrinolytic activity, is produced as a 40kDa proprotein that is processed into the 28kDa mature form [51]. The processing of the protein occurs stepwise with over eight intermediates [51, 52]. SpeB has an array of responsibilities, including degradation/cleavage of human extracellular matrix proteins, components of the immune system such as immunoglobulins and complement factors, and GAS surface proteins [53].

7.4.1 SpeB Regulation

Ultimately, the activity of SpeB allows the bacterium to evade the host immune response and disseminate from the initial infection site [39, 52]. Production of SpeB is tightly regulated, with an increase in production during late exponential and stationary phase [54]. This regulation is likely controlled by nutrient availability and culture pH. The optimum pH for SpeB synthesis falls between 6.0 and 6.5; interestingly, the pH of saliva is normally between 6.0-7.5 [22, 55, 56]. Further, artificial saliva, which was used in our studies, has a pH of 6.8-6.9. Nutrient availability also effects production of the SpeB, with increased glucose or peptides inhibiting production of the protein. The specific mechanism for how SpeB increases survivability in saliva is unknown. However, many of the host proteins and immune cells that SpeB cleaves (**Figure 4**) are typically found in human saliva. For this reason, it is likely that SpeB utilizes cleavage or degradation of a combination of host proteins and its own surface proteins to enhance survival [22, 55, 56].

7.4.2 SpeB Production and Survivability

One possible explanation of the increased survivability with possession of SpeB can be explained by the effect of SpeB on F1, a cell wall attached fibronectin (Fn)-binding protein [57, 34, 36]. F1 is present in many GAS isolates and functions in the internalization of *S. pyogenes*

into epithelial cells. SpeB degrades F1, leading to reduced internalization. Since SpeB production increases in late exponential and early stationary phase, secretion also correlates with a lack of nutrients [57, 58, 59]. When GAS lack nutrients, cleavage of F1 protein by SpeB will encourage bacterial spread to a new location with potentially more nutrient sources [58]. The use of SpeB to cleave F1 at specific times could allow the bacteria to relocate to areas with the larger quantities of nutrients before adhesion and internalization [58].

GAS uses SpeB to evade the immune response by cleaving immunoglobulins into Fc and Fab fragments, rendering them ineffective and allowing the organism to escape phagocytosis [30, 52, 60, 61]. Degradation of C3b, a component of the complement cascade that assists in phagocytosis and initiation of the alternative complement pathway, is also a function of SpeB. Additionally, SpeB releases complement factor C5a from the bacterial surface, inactivating leukocyte chemotaxis activity. C3b generates C5a and C3a, so the inactivation of C3 (C3b) by *S. pyogenes* is crucial to evade neutrophil ingestion and the production of anaphylatoxins. Destruction or inactivation of host immune components could allow *S. pyogenes* to diffuse attraction of neutrophils to the infection site and, consequently, survive for an extended amount of time in saliva [30, 52, 60, 61].

7.5 Bacterial Two Component Regulatory System, SptR/S

S. pyogenes M1 possesses 13 two-component regulatory systems (TCS), with 12 of these being highly conserved among different GAS strains [62, 63]. After comparing the expression rates of the different two component regulatory systems during growth in saliva, researchers concluded that *spy0874/0875* TCS (also called *sptR/S*) was likely linked to persistence of *S. pyogenes* in human saliva [62, 63]. Further analysis of *ΔsptR* mutants versus wild type GAS concluded that SptR/S TCR was involved in the production/regulation of many virulence factors

of GAS, including SpeB, Sic, and factors involved in nutrient acquisition, response to oxidative, and evasion of immune response [32, 44, 63]. One gene controlled by *sptR/*S is *malE,* a maltodextrin binding protein involved in nutrient acquisition and phosphotransferase [64]. The protein MalE may contribute to the ability of GAS to use carbohydrates produced from the degradation of amylase, a component of saliva [64]. TCS *sptR/S* also controls the *hasABC* operon, which is involved in capsule synthesis [51]. Proteins AmyA and AmyB are both involved in starch degradation, further illustrating the wide variety of genes and proteins regulated by TCS *sptR/S* [62]. Though little is known about the exact mechanisms of this TCS, multiple studies have illustrated the importance of the system.

One hypothesis for the contribution of SptR/S to persistence of GAS in saliva involves recognition of currently unidentified signals in saliva [63]. After this recognition, SptS undergoes autophosphorylation and subsequently phosphorylates SptR. The phosphorylation of SptR results in production/secretion of a wide range of virulence factors used in GAS survival in saliva. Additionally, phosphorylation of SptR is hypothesized to alter proteins in order to begin metabolism of carbohydrates that may affect production of GAS virulence factors [63]. This hypothesis tying SptR/S to the regulation of virulence factor production in GAS still leaves many mechanistic aspects of the TCS unexplained, and further research on this topic is needed.

7.3 Virulence Factors Linked to Colonization

After adhesion, *S. pyogenes* cells can invade epithelial cells at high frequencies, similar to that of *Listeria* spp. [23, 65]. The M protein and fibronectin binding proteins are essential to this function, and the invasion of epithelial cells has been theorized to be a mechanism for the organism's pathogenesis. In fact, the invasion is suggested to help the organism evade the host immune system and allow the infection to persist for long periods of time. Tonsils extracted from

patients with recurring tonsillitis contained intracellular GAS, strengthening this theory. Other research has led to the idea that GAS uses epithelial cell internalization to disseminate to deeper tissues.

The hyaluronic acid capsule and cytoplasmic membrane of *S. pyogenes* both help the organism to evade the host immune system. The capsule's chemical composition resembles that of host connective tissue, rendering it nonantigenic. Additionally, the cytoplasmic membrane possesses antigenic determinants similar to host tissue antigens. This allows the organism to carry out molecular mimicry. *S. pyogenes* possesses many toxins that interact with human blood. Streptolysins S and O, Hyaluronidase, Streptokinases, Sterptodornases, *S. pyogenes* pyrogenic exotoxins (SPEs), and Proteases all work together to enhance virulence of the organism and allow it to break down host immune responses. Strains possessing SPE exotoxins have been linked with severe tissue destruction and toxic shock syndrome type infections [23, 65].

7.4 Pathogenesis

S. pyogenes most commonly colonizes the oropharynx, and can colonize this site for long periods of time; inhabiting approximately one half of school age children during nonepidemic time periods [66, 31]. Most people develop GAS infections around ages 6-13. Skin infections caused by *S. pyogenes* are most common in children around 3-5 years of age. The organism typically exists as normal flora in low numbers due to competition from other microbes. However, when the normal flora is altered or another strain of *S. pyogenes* is introduced, disease can result. Upon infection, pathogenic GAS causes lesions at the site of infection, usually the upper respiratory tract, but sometimes the skin [66, 31]. Studies have shown that when a person has an *S.* pyogenes infection, between 10-25% of close contacts can also become infected [67]. Further, people who are carriers of the organism can expel 100 times as many streptococci as

someone actively sick with Strep Throat. *S. pyogenes* is documented as highly sensitive to Penicillin, however, GAS was found to remain in 35% of tonsillitis cases after treatment [68]. GAS infections of the middle ear, lungs, and sinuses can also occur. If the infection goes untreated, *S. pyogenes* can disseminate to the bloodstream, causing bacteremia [68].

S. pyogenes can be transmitted via respiratory droplets containing the organism [69]. Further, GAS can be contracted by inhalation or ingestion of these droplets through close contact with an infected person or contact with fomites containing respiratory droplets. Humans produce these droplets when breathing, coughing, sneezing, talking, or singing. Though the droplets mostly contain water, they also contain cells of the immune system and epithelial cells, mucous and other typical components of saliva including physiological electrolytes, and, potentially, infectious agents [69]. Saliva containing high concentrations of GAS is linked to person-to-person spread of the organism [20]. However, the exact infectious dose of GAS is not known. If *S. pyogenes* pharyngitis goes untreated, the infected person can be contagious for 7-10 days [70]. However, if Penicillin is administered, the organism is only transmissible for 24 hours [70].

7.4.1 *S. pyogenes* Transmission

GAS is transmitted by respiratory droplets, nasal discharge, and contact with lesions [71]. Additionally, contact with skin surfaces carrying the pathogen transmits the organism. The primary routes of spread are contact and aerosol, reflective of the modes of transmission. The possibility of airborne transmission involves droplet nuclei or particles containing the organism that are suspended in the air being inhaled. This is a very common mode of transmission for GAS due to the organism's colonization of the oropharynx. The other form of *S.* pyogenes spread is called contact transmission, and has two forms, direct and indirect contact [71].

7.4.2 Direct Contact Transmission

Direct contact is the spread of infectious disease when pathogens disseminate from the index patient to a new person through direct contact such as kissing, contact with body lesions, or contact with bodily fluids [72]. Indirect contact is the spread of infectious disease that does not involve human-to-human contact between the infected person and new person. This transmission could occur by touching a surface, such as an arm rest in the airline cabin, that became contaminated with an organism of interest when the diseased person coughed or sneezed and the airborne droplets produced infected the surface [72]. Common examples of these surfaces (called fomites) in airplane cabins are tray tables, leather chairs, window shades, and toilet buttons.

7.4.3 What is a Fomite?

The definition of a fomite is a surface or object that can become contaminated with bodily fluids including blood, mucous, urine, feces, saliva etc [73]. The surface can then serve to transmit the organism to a person. Though not all pathogens and viruses can be transmitted through fomites, many can be [73]. A study involving *Streptococcus pneumoniae* and *Streptococcus pyogenes* found that the when the bacteria were in a biofilm on a plastic fomite surface, it survived for a month to three months [74]. In addition, many viruses other than the norovirus-such as coronavirus, influenza virus, and the rhinovirus can be transmitted via fomites. Many other food pathogens are also transmitted by fomites [74].

7.4.4 The History of Fomites

Indirect contract transmission via fomites was first acknowledged in 1546 [75]. A physicist, Girolamo Fracastoro, described direct and indirect contact and speculated that porous fomites such as linen, cloth, and wood can remain contaminated for years. Though he understood that inanimate objects played some part in disease, Fracastoro did not understand that

microorganisms were ultimately responsible [75]. In later years, the knowledge of fomite transmission was used as an act of bioterrorism when British colonialists gave blankets contaminated with smallpox to Native American Indians [76].

During the time of the plague, fomite transmission was recognized with the implementation of regulations to purify fomites with suspected contamination. People believed that yellow fever was caused by fomite transmission as well, though this assumption proved to be incorrect when Dr. Walter Reed found mosquitos to be the vector. Fomite transmission was scientifically proven to be significant in the 1970s. J. Owen Hendley and Jack Gwaltney from the University of Virginia tested different transmission routes of the rhinovirus, finding that it was capable of infecting people who touched a contaminated surface. This was later confirmed by lab work suggesting the virus could be transmitted via direct and indirect contact [76].

Fomite transmission of *Streptococcus pyogenes* is a subject of conflict, and has been debated upon in the past. Some sources state that fomite transmission of GAS is inapplicable and that the organism does not remain infectious outside of the host [77]. Though this is a belief held by some scientists, there has been much research performed to indicate that *S. pyogenes* can survive outside the host for extended periods of time and that it remains infectious. *S. pyogenes* was found to survive for 3 days to 6.5 months on dry surfaces under typical atmospheric conditions [78, 79]. Further, recent studies suggest that if *S. pyogenes* is surviving in a biofilm, it can have heightened infectivity [67]. In a study conducted by L. Marks of the University of Buffalo, biofilms of *S. pyogenes* were harvested from fomite surfaces and intranasally inoculated into mice. 2 days later, the mice were found to have been colonized by GAS, indicating that the organism retains the ability to colonize mice after desiccation on a fomite surface [67].

Summary

Billions of passengers fly on airplanes every year. The close proximity in which passengers sit, potentially long flight duration, and the percentage of asymptomatic carriers of pathogens in the population all lead to documented cases of outbreaks on board airplanes. Though airplane cabin instances of transmission happen frequently, little has been done to investigate the cabin conditions in relation to the effects on proliferation and spread of bacteria. In fact, there are many surfaces on board airplanes that are frequently touched by many different passengers per day. These surfaces make ideal vessels for surface transmission, a documented route of transmission for *S. pyogenes*. With the combined ability of the organism to survive on surfaces for weeks to months, airplane cabins are a likely place of *S. pyogenes* transmission. There is a need for transmission of the organism under airplane cabin environmental conditions to be investigated further.

Figures and Tables

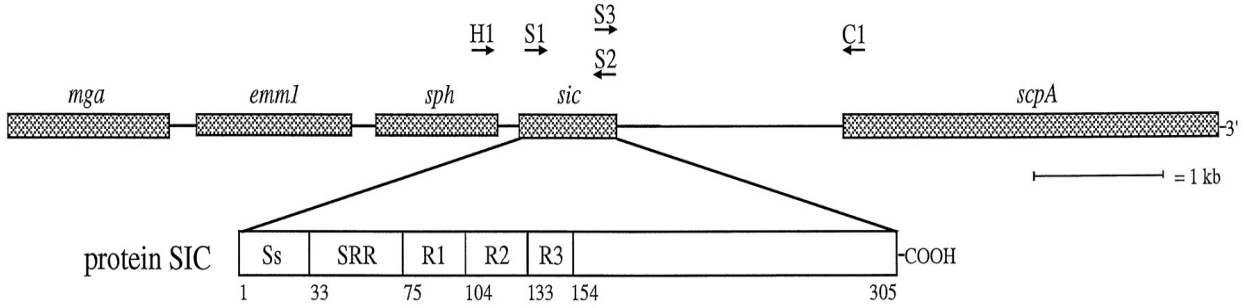

Figure 1. Map of the *mga* regulon in a M1 *Streptococcus pyogenes* strain. Gene *mga* is followed by *emm1*, *sph*, and *sic.* The components of *sic* are represented in the lower part of the figure. Taken from http://www.jbc.org/content/271/2/1081.full

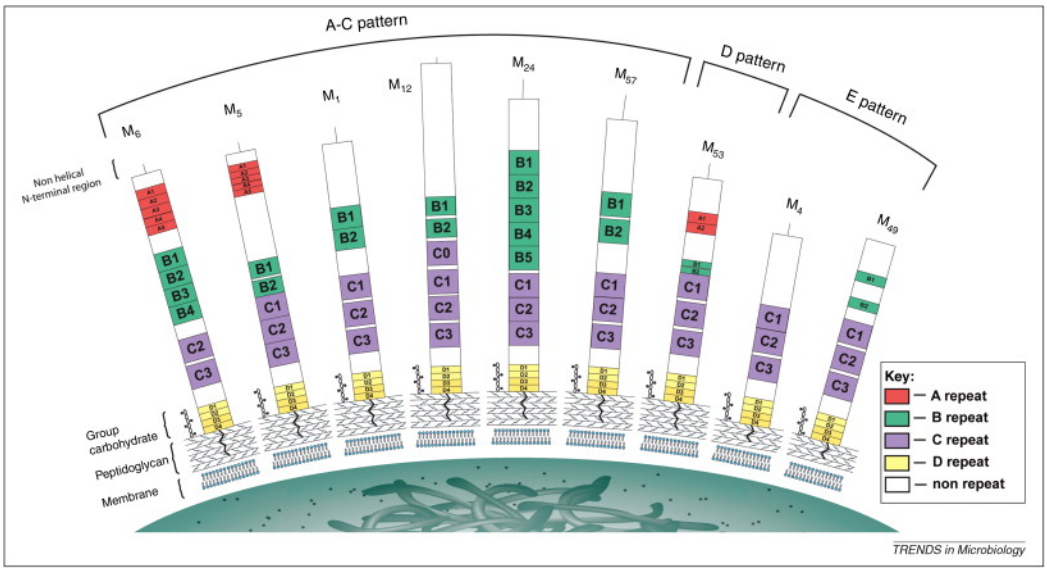

Figure 2. Comparison of differing M proteins, including M1. M1 belongs to the A-C pattern type and is larger than the other pattern types (46).

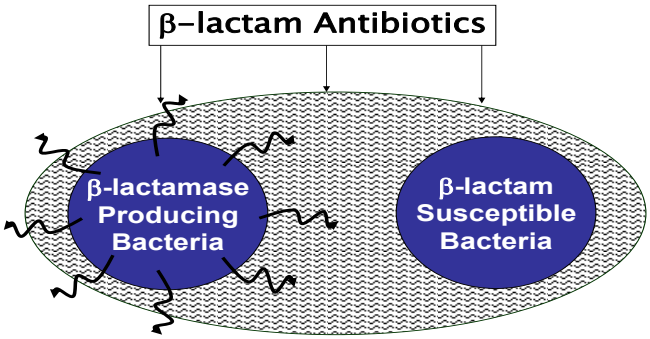

Figure 3. Illustration of the protection beta lactamase producers afford to penicillin susceptible bacteria through the release of beta lactamases into the environment (7).

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Chapter 2

Survivability and Transmissibility of *S. pyogenes* In Airplane Cabin Conditions

Abstract

We tested the survival and transmission of *S. pyogenes* (Group A *Streptococcus* or GAS), the causative agent of diseases such as Strep Throat and Necrotizing Fasciitis, on surfaces taken from airplane cabins. GAS strains and either PBS or artificial saliva were mounted onto airplane surface coupons and incubated then checked for survival. Sterile pigskin was used to determine transmissibility of *S. pyogenes* from cabin surfaces to skin. Transmission rates were determined by mounting GAS and artificial saliva or PBS onto coupon surfaces then uniformly pressing these swatches onto pigskin. GAS possessing the M1 strain survived for a minimum of 18 days, regardless of solution or coupon surface. GAS possessing the M5 strain was inhibited by saliva, surviving a minimum of 6 days. M1 strains were transmissible for a minimum of 12 days while M5 strains were for 4 days. With the typical 1-3 day incubation period of *S. pyogenes*, these findings indicate that the organism has the ability to be transmitted in airplane cabins by contact/surface transmission.

Introduction

Though an outbreak of *S. pyogenes* on an airplane has not been documented thus far, the organism poses a threat for transmission while on board. When researchers sampled an aircraft in a previous study, they cultured *S. pyogenes*, indicating the organism is capable of survival under airplane conditions [1]. Transmission is highly likely due to the prevalence of the organism and the lack of documentation is conceivably due to multiple reasons. Firstly, the organism has an incubation period of around 1-3 days [2]. Due to this and the normally mild symptoms as a result of infection, many *S. pyogenes* infections go unreported. In this thesis, the surface or contact transmissibility of *Streptococcus pyogenes* under airplane conditions is examined more closely to analyze

the risk for infection, especially with passengers in close proximity to an infected individual.

Materials and Methods

Bacterial Preparation

Throughout the study, two strains of *S. pyogenes* were used. The first, *S. pyogenes* Rosenbach ATCC® 19615, was initially isolated from the pharynx of a child following an episode of sore throat [3]. The strain possesses the M5 protein and is used as a reference strain in many quality control tests [4]. The second strain, *S. pyogenes* Rosenbach ATCC® BAA-947D, possesses the M1 protein and is heavily characterized [2, 21, 22, 23]. This strain is used in research involving *S. pyogenes* survival in saliva. Both strains were initially stocked in 20% (v/v) glycerol and maintained at -80°C. The strains were resuscitated separately in 10mL of Todd Hewitt Broth (THB), and incubated until mid log phase ($OD_{600} = 0.7$) at 37°C, without shaking. Streak plates were made from both cultures on Sheep Blood Agar (SBA). To confirm GAS, beta hemolysis was observed on the SBA plates and a latex agglutination test was conducted using

one colony from the plates. Both master plates were stored at 25°C as *S. pyogenes* does not survive well at 4°C.

To determine the correlation between growth time, OD_{600} reading, and CFU/mL counts, a growth curve was conducted. An OD_{600} reading of 1.0 was found to correlate to 10^8 CFU/mL at 10 hours of incubation and late log phase. This reading was confirmed by multiple journal articles and used throughout the rest of the research to quantify the amount of bacteria per surface used [5, 6, 7, 8]. To prepare the two GAS strains for the survivability and transmissibility experiments, a colony was selected from a GAS master plate of the appropriate strain and suspended in THB. The cultures were then incubated for 10 hours without shaking at 37°C. After incubation, these cultures were centrifuged at 6000 rpm for 10 minutes to remove the growth medium. The supernatant was removed and the pellet was re-suspended in either PBS or saliva. This centrifugation and subsequent re-suspension was repeated three times before the final suspension in PBS or artificial saliva to remove any remaining nutrients. The OD was adjusted to 1.0 using PBS or saliva and the CFU/mL cell concentration of $1x10^8$ was confirmed through serial dilution and plating.

Airplane Cabin Surfaces

The surfaces used in this study were samplings of four different surfaces taken from an airplane cabin. These surfaces included: stainless steel toilet button, rubber armrest, plastic tray table, and plastic window shade. The surfaces were cut into 1x1 cm coupons and sterilized initially by gamma irradiation. To maintain sterilization, coupons were autoclaved and a control of each surface was incubated to confirm the absence of bacteria. For survivability studies, 3 coupons of each surface per strain of GAS and suspension were monitored every 48 hours per variable for a maximum time period of 30 days.

Pigskin

Gamma irradiated pigskin (Leach Nuclear Science Center, Auburn University, AL) was used as a surrogate for human skin to determine the transmissibility of *Streptococcus pyogenes* from airplane cabin surfaces under cabin conditions to skin. The pigskin was obtained from Dean Sausage Company (Atalla, AL). It was washed, shaved, and cut into 1x1cm swatches then exposed to gamma irradiation for 1, 330 minutes or 22 hours. The cobalt 60 irradiating source was exposed at a dose of 3.065 Mrad. Using gamma radiation to sterilize skin has been previously documented as successful [9]. A pigskin swatch was taken from all bottles used after irradiation and incubated in a growth medium to confirm sterilization.

Survivability Studies Using Airplane Cabin Coupons

Overnight cultures of *S. pyogenes* with M1 and M5 proteins were pelleted, washed to remove nutrients, then re-suspended in either phosphate buffered saline (PBS) or saliva (Arnof NF, Pickering labs, Mountain View CA). Cultures were sonicated (Sonicator Q700, Qsonica, LLC Newtown, CT) for 4 10-second bursts to break the characteristic chain arrangement of *S. pyogenes* that can confound CFU/mL counts [10]. *S. pyogenes* divides in one plane, accounting for the chain like arrangement and making them difficult to separate [11].

OD_{600} values were obtained and adjusted to a 1.0 optical density reading (Genysys 20 spectophotometer, Thermoscientific,Waltham, MA). The CFU/mL cell concentration of $1x10^8$ was confirmed through serial dilution and plating. Since two strains were used in either PBS or Saliva on four different surfaces, the different survivability experiments conducted were as shown in **Table 1**.

After obtaining an OD_{600} value of 1.0, 25µL of the suspension was mounted to 47 coupons per surface, per strain and suspension, giving an initial inoculum of $2.5x10^6$ CFU/mL

onto each coupon. Immediately after drying of the bacterial suspension on the coupon (approximately 60 minutes), 2 coupons per surface were removed and added to 5mL of sterile, filtered 1x PBS then sonicated for 20 seconds at 2 W, 20 KHz, 50 amp. Sonication under these conditions had previously shown optimal survival. The 5mL PBS containing each coupon was then vortexed for an additional 10 seconds, diluted, and plated in duplicates on Sheep Blood Agar (SBA) to determine CFU/mL cell counts. To confirm GAS, beta hemolysis was observed and a latex agglutination test was performed on randomly selected colonies. The remaining coupons were incubated in an environmental humidity chamber (Caron 6020, CARON Products & Services, OH) with approximately 18% relative humidity at 27°C.

Three coupons per surface were removed, sonicated, vortexed, diluted, and plated in duplicates at 48-hour intervals until no cell counts were obtained. When no colonies were observed on a plate, the strain was considered non-culturable. The experiment was repeated to give results for a total of 6 different coupons per surface per strain and suspension for each time point. Overall, M1 GAS with PBS, M1 GAS with artificial saliva, M5 GAS with PBS, and M5 GAS with artificial saliva were tested to determine survivability [12, 13, 14].

Transmission Studies Using Airplane Cabin Coupons

Overnight cultures of *S. pyogenes* with M1 and M5 proteins were pelleted then re-suspended in either phosphate buffered saline (PBS) or artificial saliva. Cultures were sonicated for 4 10-second bursts to break the characteristic chain arrangement of *S. pyogenes*. OD_{600} values were obtained and adjusted to 1.0 using a spectrophotometer. The CFU/mL cell concentration of $1x10^8$ was confirmed through serial dilution and plating. Since two strains were used in either PBS or Saliva on four different surfaces, the different survivability experiments conducted were according to the **Table 1**.

25μL of the appropriate suspension was mounted to 32 coupons per surface per GAS strain, per suspension. After drying, 2 coupons from each surface were removed and initial counts were obtained by pressing a 1x1cm coupon of gamma irradiated pigskin to each cabin surface coupon a for 3 seconds using a compressing apparatus (Sargent Welch, Serial No: 342104 Catalog No:23207, Rochester, NY) to apply equal amounts of pressure. After applying pressure, the pigskin swatches were immediately removed with sterile forceps and each added to an individual 5mL of PBS then vortexed for 30 seconds. This suspension was next diluted and plated on SBA in duplicates to obtain CFU/mL cell counts. Two coupons per surface were removed, pressed with pigskin, vortexed, diluted, and plated at 24-hour intervals until no viable cell counts were obtained. The experiment was repeated to give results for a total of 4 different coupons per surface and solution for each time point. Overall, M1 GAS with PBS, M1 GAS with artificial saliva, M5 GAS with PBS, and M5 GAS with artificial saliva were tested to determine transmissibility.

In addition, a control swatch of pigskin was incubated in THB. 25μL of each strain and one of the two suspensions (artificial saliva or PBS) was also mounted onto pigskin. The pigskin swatches representing M1 GAS and PBS, M1 GAS and artificial saliva, M5 GAS and PBS, and M5 GAS and artificial saliva were vortexed in 5 mL PBS, diluted, and plated to obtain a baseline range for the transmission experiment.

Results

Statistical analysis utilizing the chi test to determine p values in Microsoft Office Excel showed that there is a significant difference among survival of M1 strains on the different surfaces and with exposure to the different solutions ($p < .05$). This was also true when using the M5 strain of *S. pyogenes* ($p < .05$)

M1 Survival Studies

Coupons mounted with M1 GAS and PBS were assessed for survivability until no viable cells were detected. GAS possessing the M1 protein and suspended in PBS survived for 432 hours or 18 days on the plastic window shade. Survival on the polyurethane armrest, plastic tray table, and window shade was 576 hours or 24 days. The bacteria survived the longest on the toilet button, armrest, and tray table when suspended in PBS. It survived for the least amount of time on the plastic window shade. The bacteria existed in the highest concentration at 24 hours on the armrest surface.

Coupons mounted with M1 GAS and artificial saliva were checked for survivability until no viable cells were detected. By using standard plate count methods, it was determined that GAS possessing the M1 protein suspended in artificial saliva survived for 432 hours or 18 days on the window shade. The organism survived for 528 hours, or 22 days on the tray table, toilet button, and polyurethane armrest surfaces. The bacteria survived the longest on tray table, armrest, and toilet button and for the least amount of time on the window shade when suspended in artificial saliva. The bacteria existed in the highest concentration at 22 days on the armrest surface.

When the data from M1 survivability in PBS and M1 survivability in artificial saliva, the results were similar as seen in **Figures 4 and 5**. The toilet button surface GAS survived for 22-24 days. The window shade coupon yielded viable *S. pyogenes* for 18 days in both solutions. The other two surface showed similar survival in both solutions within a 48 hour range.

M1 Transmission Studies

S. pyogenes strains possessing the M1 protein were monitored for transmissibility as depicted in **Figures 6 and 7**. After monitoring coupons with strains exposed to artificial saliva every 48 hours, CFU/mL counts were compiled. GAS mounted to the armrest and plastic window shade

were detected for the least amount of time, 288 hours or 12 days. Organisms on the metal toilet button were transmissible for 336 hours or 14 days. The plastic tray table was transmitted for the longest amount of time, 528 hours or 22 days. The armrest facilitated transmission of *S. pyogenes* for the second longest amount of time, 18 days.

S. pyogenes strains with the M1 protein were also exposed to PBS, and these coupons were tested for transmissibility every 48 hours. The CFU/mL counts revealed that GAS was transmissible on the plastic tray table the longest, for 480 hours or 20 days, under the specified conditions. The plastic window shade and toilet button retained viable, transmittable GAS for 288 hours, or 12 days. The polyurethane armrest only facilitated transmission for 8 days, or 192 hours.

To assess the functionality of the transmissibility model, a $2.5x10^8$ CFU/mL cell concentration of the M1 strain was suspended in either PBS or artificial saliva and mounted on a swatch of pigskin. $4x10^5$ CFU/mL was recovered when suspended in saliva and $5.1x10^5$ CFU/mL was recovered when suspended in PBS, 16% and 20% of the inoculum, respectively. When pigskin swatches alone were incubated, no GAS or contamination was observed.

M5 Survival Studies

As seen in **Figures 8 and 9,** *S. pyogenes* strains possessing the M5 protein were monitored every 48 hours until no viable cells remained; thus determining survivability. M5 GAS exposed to artificial saliva was monitored on each of the four surfaces, and CFU/mL values were compiled for analysis. Overall, the surfaces had a very similar rate of survival. Survival on any surface did not extend beyond 6 days, or 144 hours. However, GAS on the plastic window shade and tray table survived for 96 hours, or 4 days. On the other two surfaces, the toilet button and polyurethane armrest, GAS survived for slightly longer, 6 days.

M5 GAS exposed to PBS was also monitored on all four coupon surfaces. Plate counts revealed that GAS remained culturable when mounted to the plastic window shade for 336 hours, or 14 days. M5 GAS survived on the toilet button for 384 hours, or 16 days. The organism survived on the polyurethane armrest and tray table surfaces for 432 hours, or 18 days. M5 GAS exposed to PBS and saliva were also compared. Overall, M5 strains survived for 10 days longer when exposed to PBS instead of artificial saliva.

M5 Transmission Studies

S. pyogenes strains possessing the M5 strain mounted onto the 4 coupon surfaces were monitored for transmissibility every 48 hours, as illustrated in **Figures 10 and 11**. The strain did not survive for longer than 6 days in the presence of artificial saliva. Specifically, all surfaces contained GAS when monitored at 48 hours, but the strains were dead at 96 hours.

M5 GAS strains exposed to PBS on the polyurethane armrest coupon survived for longer than 528 hours, or 22 days. The tray table coupons also exhibited survival for 22 days. On the other hand, GAS mounted onto the toilet button and window shade surfaces survived for 384 hours, or 16 days.

To assess the functionality of the transmissibility model, a $2.5x10^8$ CFU/mL cell concentration of the M5 strain was suspended in either PBS or artificial saliva and mounted on a swatch of pigskin. $1.3x10^6$ CFU/mL was recovered from the M5 suspension with saliva, approximately 52% of the initial inoculum. $1.5x10^6$ CFU/ML was recovered from the M5 suspension with PBS, approximately 60% of the initial inoculum. When pigskin swatches alone were incubated, no GAS or contamination was observed. These numbers were used to calculate transmission rates of M1 and M5 GAS as pictured in **Table 2 and 3.**

Discussion

Norovirus and *N. meningitidis*, two disease causing agents with documented outbreaks on airplanes, can both be transmitted by contact transmission [15, 16, 17, 18]. *S. pyogenes*, also with the ability to be transmitted by contact, is likely transmitted on airplanes [19]. The large amount of asymptomatic carriers and frequency of infection of the organism makes *S. pyogenes* an ideal pathogen for survival and transmission studies on surfaces [20, 17]. Survival studies have been performed for GAS on fomite surfaces and the extended length of the organism's survival is well documented [2, 21, 22, 23]. However, none of these studies were conducted using the conditions that mimic an airplane cabin environment, with a low relative humidity (around <18%) and a temperature around 18-24°C. Our studies analyzed the survival and transmission of the pathogen when exposed to these conditions. In addition, we determined the effects of artificial saliva on the survival and transmission of GAS, since the organism's most prominent colonization site is the oropharynx [24].

A combination of direct sonication and vortexing was used to obtain the bacteria from the coupon surfaces. This was used as opposed to soaking, swabbing, or scraping the surfaces due to a larger yield of bacteria after sonication. Though swabbing surfaces is a very common practice in environmental work, it lacks sensitivity due to a tendency of incomplete bacterial load transfer from the cotton fibers to the growth media [25]. Further, sonication is a proven superior method for bacterial retrieval [26].

The *S. pyogenes* strain containing the M1 protein was used because of its ability to survive and proliferate in saliva as well as its virulent nature. The M5 strain was used as a comparison strain. When placed in PBS, the survival of the M1 and M5 strains was relatively similar. Overall, the polyurethane armrest surface facilitated survival for the longest amount of time. The armrest was the only porous surface used in the study. Extended survival on the armrest was

observed in both M1 and M5 strains when exposed to saliva and PBS. We hypothesize that survival on the porous surface is due to the additional protection that the surface may afford to the bacteria. The pathogen may colonize within the indentions of the surface, allowing it a more favorable environment to possibly grow in a biofilm and be protected from elements such as desiccation. Biofilm formation is proven to enhance *S. pyogenes* fomite survival and could contribute to the extended survival of the organism [22]. The survival of bacteria on porous surfaces like hospital sheets has been previously documented as significant [27]. Overall, the polyurethane armrest surface facilitated the highest rates of GAS survival, second to the tray table. Though nonporous, the tray table surface does have indentions and ridges. This could also provide the bacteria with a niche in which it could better survive. Survival was generally the least on the toilet button and window shade surfaces. These surfaces are a nonporous. The toilet button surface is a nonmetal surface made of stainless steel. Studies to determine survivability on stainless steel indicated that the surface does not have known antimicrobial effects [28]. We hypothesize that the decreased survivability is due to the lack of ridges or indentations of the surface, leading to more rapid desiccation and decreased viability of the organism. The window shade surface also lacks ridges or indentations. This could contribute to its decreased facilitation of survival.

M1 strains exhibited similar survival of around 22 days, regardless of the suspending media. This is likely attributed to the possession of the M1 protein, including possession of the *sic* and *speB* genes. These proteins and genes allow the organism to survive and proliferate in saliva. M5 strains lack the M1 protein and possesses little SpeB [6]. M1, on the other hand, produces an abundance of SpeB. Consequently, the presence of the protein is linked to GAS survival in saliva [13]. Streptococcal Inhibitor of Complement, or Sic, is only found in GAS types M1 and M57.

The presence of this protein is also linked to survival in saliva. Since, M5 possessing GAS strains lack these factors, survival in saliva rapidly decreases as observed in our study on all surfaces ($p < .05$). Though survival of M5 strains on the armrest surface was prolonged, all surfaces lacked viable counts after six days. Strains of GAS possessing the M5 protein are commonly linked to cases of rheumatic fever [29, 30]. Rheumatic fever is the result of an untreated case of scarlet fever or strep throat that has an effect on the heart, skin, brain, and joints [31]. This disease results around 14 days after the initial strep throat infection. Since M5 GAS survived for at least 6 days on the coupon surfaces, this could align with the dissemination time in a rheumatic fever case since desiccation is an additional stress that limits survival [22].

Transmission studies were conducted using gamma irradiated pigskin as a surrogate for human skin. Human skin was not used due to the need for the skin surface to be tested under the same conditions for a month-long period. Additionally, we wanted the amount of pressure applied to the pigskin and coupon when transferring the bacteria to be as uniform as possible. This would be very difficult with human volunteers. We elected to utilize pig skin for the studies due to availability and similarity to human skin [32]. We used the methods outlined by Maish, et al. (2007) to determine rates of transmission from coupon surfaces to skin. Gamma irradiation of the pigskin was chosen because of its acknowledgment as a method of complete sterilization and use to disinfect pigskin in hospital settings [33].

The tray table surface facilitated transmission of bacteria from the coupon to the pigskin for the longest amount of time. This finding was consistent in both M1 and M5 GAS studies ($p < .05$). We hypothesized that the transmission was consistent with the tray table being a nonporous surface and its ability to facilitate the survival of GAS. The nonporous nature of the tray table likely increased transmission rates. Porous surfaces that created a niche inside of crevices for the

organism to survive in may transfer less bacteria to the pigskin due to attainability. Non-porous surfaces, contrarily, have a more uniform surface free of crevices. Additionally, survivability studies proved the tray table surface facilitated survival longer than the toilet button and window shade surfaces due to the indentions and ridges that could have encouraged biofilm formation. The porous polyurethane armrest surface possessed the second longest transmission times, next to the tray table. Though the armrest coupon was porous, viable cells were harvested from the surface for much longer than the toilet button or window shade. This could translate to a longer transmission time from the armrest to pigskin.

As with the survivability studies, the transmission model yielded counts with the M1 GAS suspended in PBS and artificial saliva for a similar amount of time, as illustrated in **Table 5**. Also, in accordance to the survivability studies, transmission of the M5 GAS strain suspended in saliva was limited due to the inability of the strain to survive in saliva. However, when the M1 and M5 bacterial strains were inoculated onto separate pigskin swatches and plate counts were obtained, M5 GAS plate counts were higher than M1 plate counts. Possible explanations for the higher transmission rates of M5 *S. pyogenes* vs M1 *S. pyogenes* could be due to lessened growth of M1 as a result of oxygen sensitivity. Due to the facultative anaerobic and 5-10% CO_2 preferences of the organism, survival in ambient atmospheric conditions can limit growth of the pathogen [34]. Since M1 strains did survive when dried on the coupons for longer than the M5 strains, this is likely not the case. The most probable explanation could be be explained with the pigskin. M5 strains may be more sensitive to the proteins contained in pigskin, using them as nutrients to continue growth. Additionally, M5 strains may be more sensitive to desiccation than the M1 strains, indicated by the diminished overall survival of M5 GAS.

Though our experiments yielded data with visible trends and significance, they were not devoid of confounding results and discrepancies. When comparing M1 and M5 survival in PBS, M1 survived for ~100 hours longer than M5 strains. SpeB activity and production, a key characteristic of M1 GAS that allows it to survive in saliva, is actually detrimental to biofilm formation [35]. Biofilm formation has a strong correlation with the ability to survive dessication [22]. Since M5 GAS lacks SpeB, the extended survival of M1 is confounding. The survival could possibly be due the greater success of M1 as a pathogen. This could indicate that M1 GAS is a stronger, more stable strain of the bacteria.

We used an apparatus to evenly apply pressure to the pigskin and coupon when transferring the bacteria. However, this apparatus did not allow us to apply the same amount of pressure every time the experiment was conducted. Further, in a real-life scenario, the amount of force exerted on a contaminated surface by a person could vary wildly. We pressed the coupon to the pigskin for 3 seconds, however, the contact time when a human touches a contaminated surface also varies. Though the pressure and time did allow for transmission of the bacteria from the coupon to the pigskin, it is not certain that this model would yield the same results when determined using a human surrogate. If the resources were present, a further study using a human example instead of pigskin could prove beneficial.

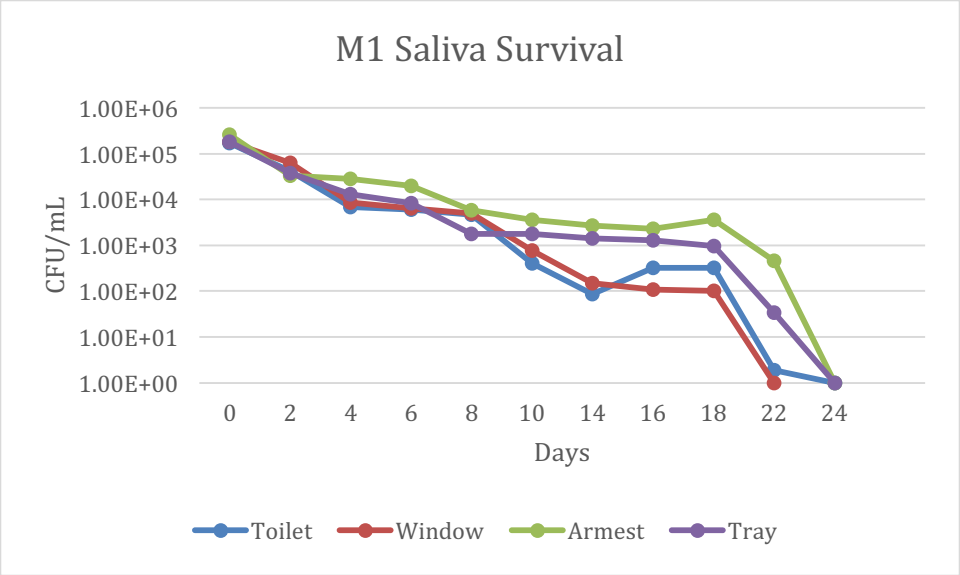
Further, the chain like arrangement of *S. pyogenes* is difficult to disrupt. Only after sonication could a uniform CFU/mL correlating to an OD_{600} of 1.0 be obtained. Even then, there were still varying initial inoculums at times. Also, *S. pyogenes* is a nutritionally fastidious organism that is sensitive to its environment. When grown on nutrient rich media, *S. pyogenes* only exhibited typical growth patterns (upon being cultured) for 4-6 days after incubation at room temperature. The organism did not tolerate refrigeration or shaker incubation. This resulted in a great deal of

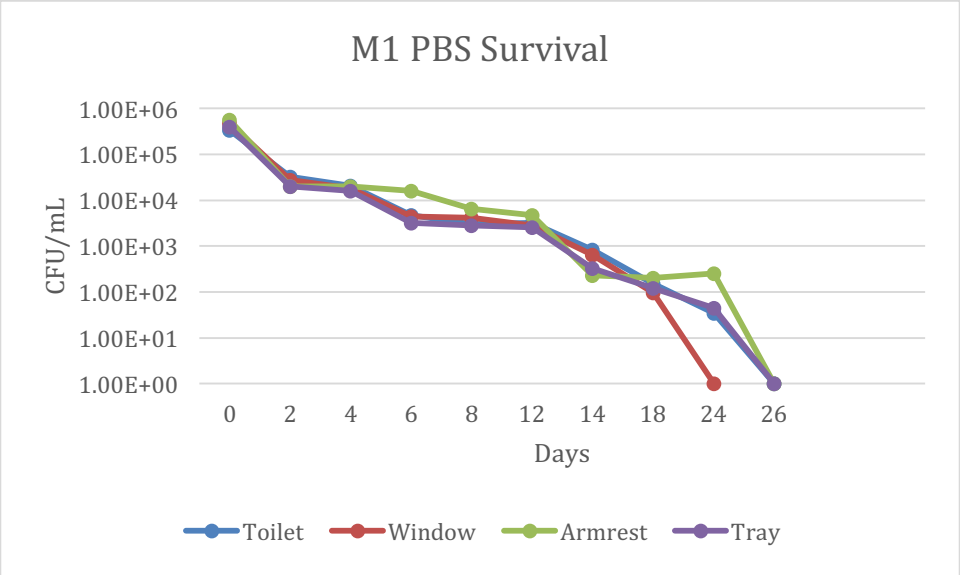
troubleshooting at the beginning of the project. However, after the ideal growth conditions and procedures for the organism were determined, GAS was able to produce a moderately uniform pattern of growth.

Conclusion

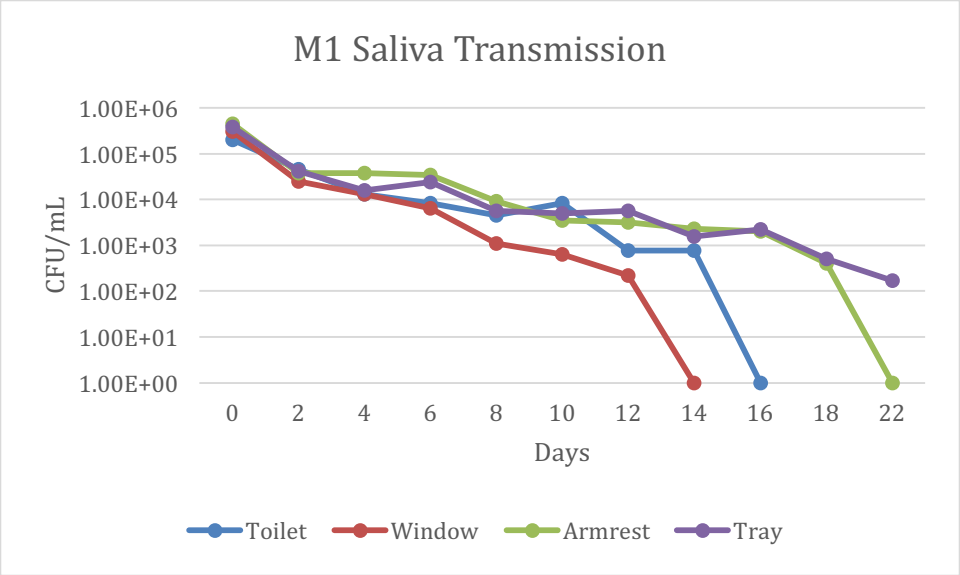
Studies were conducted to determine the survival and transmission times of both M1 and M5 *S. pyogenes* on airplane surfaces under airplane cabin conditions. The two strains were also subjected to PBS and artificial saliva suspending mediums to analyze growth. We concluded from the studies that the survival of *S. pyogenes* under airplane cabin conditions was significant. With a minimum survival of 6 days and the maximum up to 24 days, the lack of disinfection protocols and continued survival of the pathogen on airplane surfaces must be addressed. Though the infectious dose of GAS is unknown, it is thought to be low (10^3) [36]. Some conditions and surfaces retained this concentration of bacteria for up to 18 days. Previous studies have validated the infectivity of desiccated biofilms of the organism, indicating that strep remains capable of causing disease under similar conditions [22]. These findings provide further evidence for the contact transmission of *S. pyogenes* on board of airplanes and reiterate the need for proper disinfection protocols in airplane cabins.

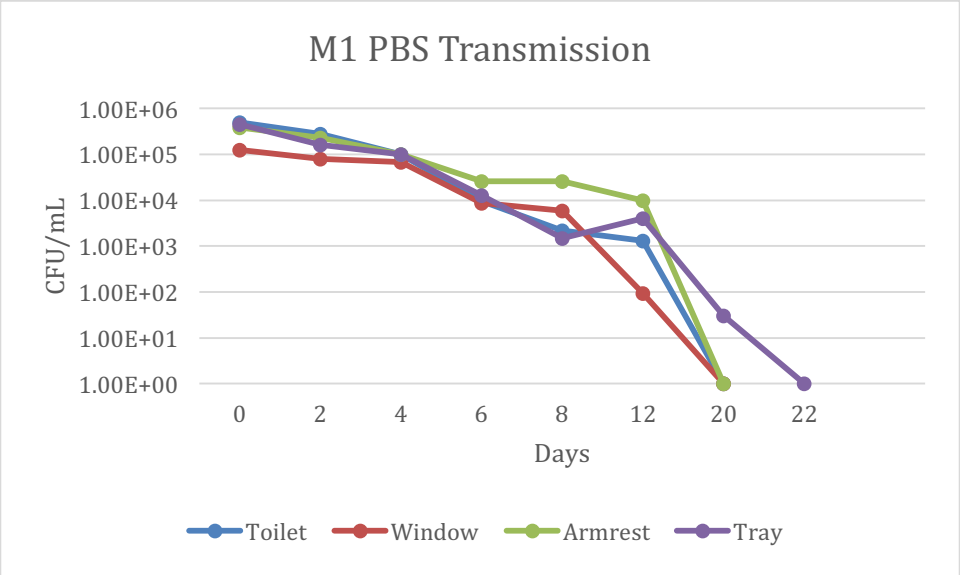
Figures and Table



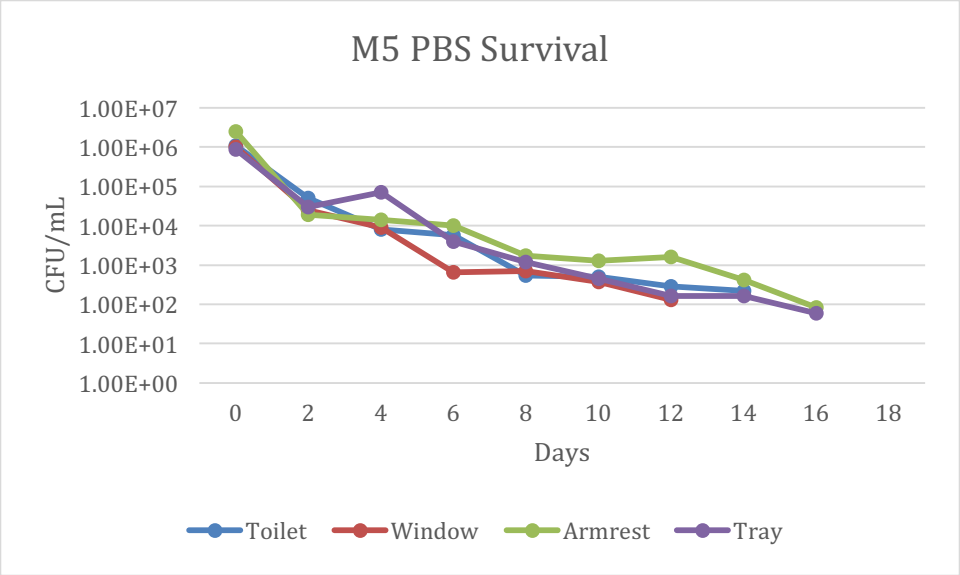


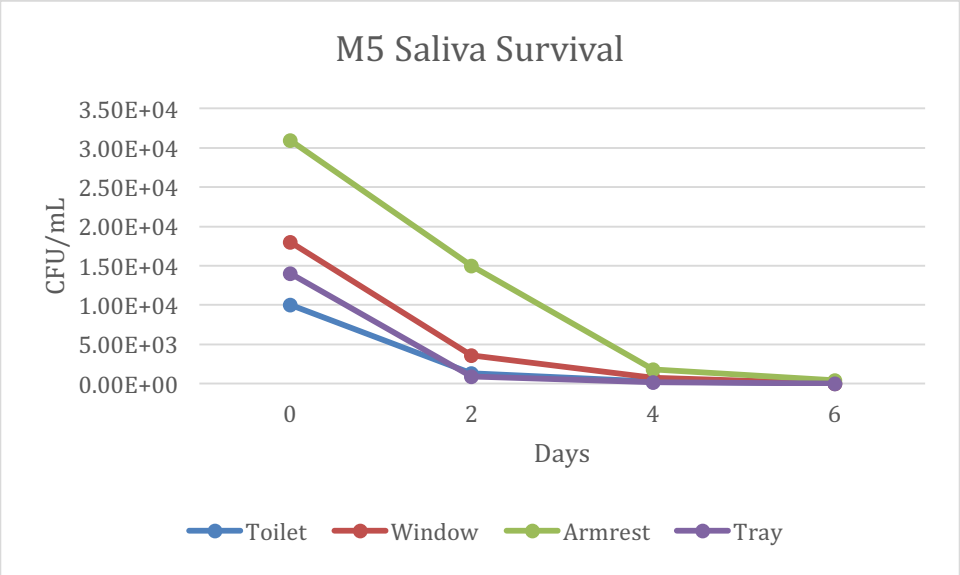
Figures 4 and 5. Representations of the CFU/mL survival counts obtained for GAS with the M1 protein and PBS or artificial saliva.



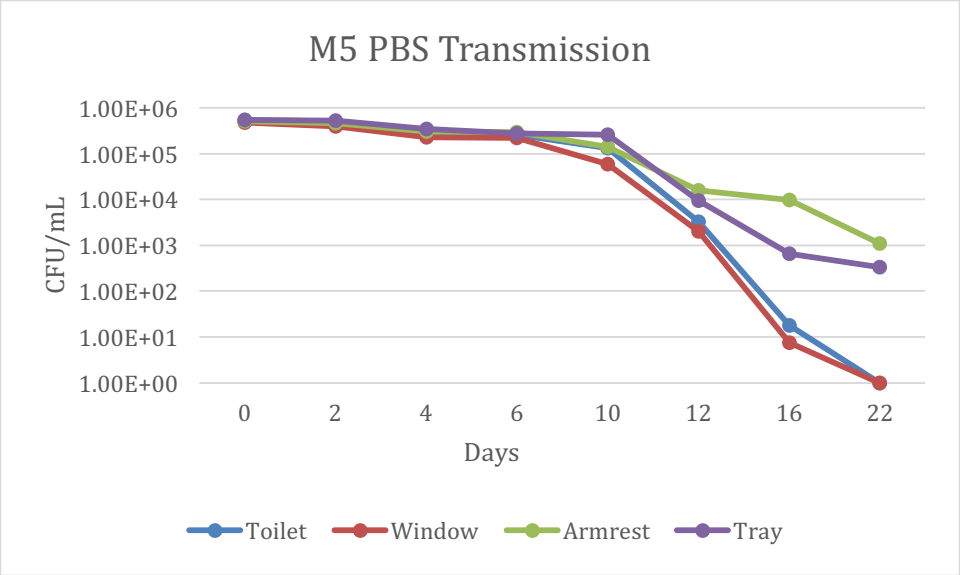


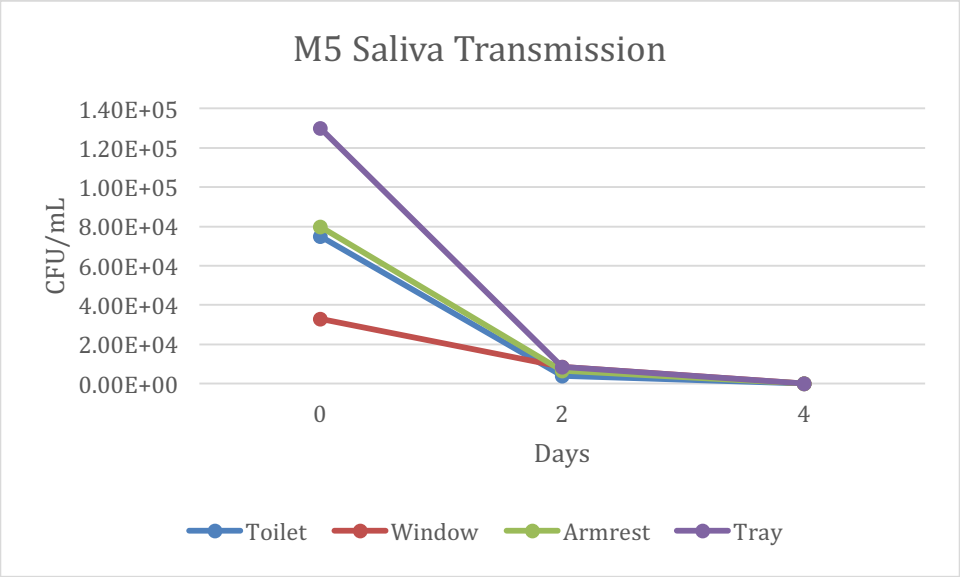
Figures 6 and 7. Representations of the CFU/mL transmission counts obtained for GAS with the M1 protein and PBS or artificial saliva.





Figures 8 and 9. Representations of the CFU/mL survival counts obtained for GAS with the M5 protein and PBS or artificial saliva.





Figures 10 and 11. Representations of the CFU/mL transmission counts obtained for GAS with the M1 protein and PBS or artificial saliva.

Experimental Conditions

Suspending Media	Strain of GAS	Coupon Surface
PBS	M1	Tray Table
PBS	M1	Toilet Button
PBS	M1	Rubber Armrest
PBS	M1	Window Shade
Artificial Saliva	M1	Tray Table
Artificial Saliva	M1	Toilet Button
Artificial Saliva	M1	Rubber Armrest
Artificial Saliva	M1	Window Shade
PBS	M5	Tray Table
PBS	M5	Toilet Button
PBS	M5	Rubber Armrest
PBS	M5	Window Shade
Artificial Saliva	M5	Tray Table
Artificial Saliva	M5	Toilet Button
Artificial Saliva	M5	Rubber Armrest
Artificial Saliva	M5	Window Shade

Table 1. A depiction of all the different variables and experiments conducted for the survival and transmission studies.

M1 Transmission

Surface	Media				Time				
		0 hr.		**144 hr.**		**288 hr.**		**480 hr.**	
		Average CFU	**%**	**Average CFU**	**%**	**Average CFU**	**%**	**Average CFU**	**%**
Toilet	**PBS**	5.00E+05	98%	9.60E+03	1.80%	1.30E+03	0.30%	0.00E+00	0.00%
	Saliva	2.00E+05	50%	8.20E+03	2.10%	7.80E+02	0.20%	0.00E+00	0%
Window	**PBS**	1.25E+05	25%	8.70E+03	1.70%	9.50E+01	0.02%	0.00E+00	0%
	Saliva	3.00E+05	75%	6.50E+03	1.60%	2.20E+02	0.06%	0.00E+00	0%
Armrest	**PBS**	3.80E+05	75%	2.60E+04	5.90%	1.00E+04	2.00%	0.00E+00	0%
	Saliva	4.40E+05	110%	3.40E+04	8.50%	3.20E+03	0.80%	4.00E+02	0.10%
Tray	**PBS**	4.50E+05	88%	1.30E+04	2.50%	4.00E+03	0.78%	3.00E+01	0.13%
	Saliva	3.80E+05	33%	2.40E+04	6%	5.70E+03	1%	5.00E+02	0.13%

Table 2. Transmission rates of M1 possessing *S. pyogenes* on airplane cabin surfaces under airplane environment conditions. Four surfaces and two suspending medias were analyzed at different timepoints. To obtain the transmission percentages, the following formula was used:

$$\% \text{ Transmission} = \frac{\text{CFU / mL recovered from pigskin}}{\text{CFU / mL recovered from coupon}} \text{x}100$$

M5 Transmission

Surface	Media				Time				
		0 hr.		**144 hr.**		**288 hr.**		**528 hr.**	
		Average CFU	**%**	**Average CFU**	**%**	**Average CFU**	**%**	**Average CFU**	**%**
Toilet	**PBS**	1.10E+06	73%	2.60E+05	17.00%	3.30E+03	0.22%	0.00E+00	0.00%
	Saliva	7.50E+04	6%	0.00E+00	0.00%	0.00E+00	0.00%	0.00E+00	0
Window	**PBS**	1.40E+06	93%	2.20E+05	15.00%	2.00E+03	0.13%	0.00E+00	0
	Saliva	3.30E+04	3%	0.00E+00	0.00%	0.00E+00	0.00%	0.00E+00	0
Armrest	**PBS**	1.10E+06	73%	2.90E+05	57.00%	1.60E+04	1.10%	1.10E+03	0.1%
	Saliva	8.00E+04	6%	0.00E+00	0.00%	0.00E+00	0.00%	0.00E+00	0
Tray	**PBS**	9.80E+05	65%	2.80E+05	55.00%	9.50E+03	1.90%	3.30E+02	0.01%
	Saliva	1.30E+05	10%	0.00E+00	0%	0.00E+00	0%	0.00E+00	0.00%

Table 3. Transmission rates of M5 possessing *S. pyogenes* on airplane cabin surfaces under airplane environment conditions. Four surfaces and two suspending medias were analyzed at different timepoints. To obtain the transmission percentages, the following formula was used:

$$\% \text{ Transmission} = \frac{\text{CFU / mL recovered from pigskin}}{\text{CFU / mL recovered from coupon}} \text{x}100$$

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Chapter 3

The Disinfection of Airplane Cabins

Literature Review

3.1 Airplane Disinfection

Due to numerous documented outbreaks and typical airplane cabin conditions, protocols used to remove infectious agents from plane cabins are in great need of development and recognition. The more flights an aircraft can complete per day; the more airline companies can maximize profits. The resulting larger movement of passengers and quick flight turnaround times make it more difficult to completely disinfect an aircraft in between every flight. Further, since passengers would almost immediately re-board the airplane after disinfection, harsh chemicals or irritants must always be avoided. Ultimately, a unique combination of a time efficient and mild disinfection protocol need to be developed for use on aircrafts. The following review will characterize different types of recommended disinfectants as defined by the Centers for Disease Control (CDC), their known efficacies, and potential practical methods of application.

3.1.1 Current Airplane Disinfection Protocols

Per the WHO Guidelines for Hygiene and Sanitation in Aviation, only a general cleaning guideline is published and not strictly enforced [1]. This guideline states that, "For operations having short flights, minimum service and short turnaround time, the requirement for cleaning between sectors is limited to very few of the procedures mentioned in the chart below". The chart referred to describes the various surfaces and areas within an airplane cabin and recommendations for cleaning. Listed in **Table 4**, the chart dictates that only overnight should passenger areas of the airplane be cleaned, with the exception of the bathroom area [1].

Since many bacterial species have been found to be capable of survival on fomite surfaces for prolonged periods of time, this cleaning schedule allows for a passenger infected with a pathogen capable of contact transmission to spread the organism to every person that contacts a contaminated surface until the plane stops for the night. For these reasons, there is a great need for a uniform disinfection protocol including a list of disinfectants. To understand the reasoning behind the WHO's recommended disinfectants for airplane cabin use, it is necessary to be familiar with the different types of disinfectants and classification schemes used when discussing disinfection.

3.1.2 Types of Disinfectants

Disinfection is the elimination of all pathogenic microorganisms with the exception of spores [2]. It is different from sterilization, which kills everything including spores. However, some disinfectants, called chemical sterilants, can kill spores within 3-12 hours. There are 3 levels of disinfectants: high, intermediate, and low. High level disinfectants kill all microorganisms except for spores within 20 minutes. Intermediate level disinfectants can eliminate hard to kill organisms like *Mycobacterium spp.* but not always spores. Low level disinfectants kill most vegetative bacteria and some fungi and viruses in less than 10 minutes.

Disinfectants are typically too harsh to be used on skin or tissues, posing a problem for airlines in the event a disinfectant comes in contact with a passenger. However, certain disinfectants are still recommended due to their ability to kill bacteria, fungi, and viruses. This is important because the type of organisms brought on board airplanes by passengers are rarely known [2].

3.1.3 WHO Recommended Disinfectants

The WHO published guide recommends using hydrogen peroxide and ethanol based disinfectants in airplane cabins. Ethanol, or ethyl alcohol, is active against fungi, vegetative bacteria, and some viruses [3]. Ethanol is most effective when used in a 70% volume per volume concentration with water and an exposure time of ten minutes is necessary for killing. Ethanol does not kill spores, some viruses, or *Mycobacterium tuberculosis*. It is also extremely flammable and can dissolve certain glues and coatings [3].

Hydrogen peroxide is active against bacteria (including *M. tuberculosis*), fungi, yeasts, viruses and spores [4]. The exposure time of the disinfectant ranges from 1 minute for most bacteria and some viruses to 60 minutes for spores. A 10% concentration of hydrogen peroxide is recommended for most efficient killing. Once the seal on a bottle of hydrogen peroxide is broken, the peroxide is exposed to air and begins to react to form water. Consequently, the shelf life is reduced to 30-45 days. The reduced shelf life and frequent need for replacement of hydrogen peroxide likely makes it less appealing as a disinfectant for airline companies [4]. Additionally, hydrogen peroxide can be corrosive to some metals and decolorize fabrics and skin [3].

3.1.4 Quaternary Ammonium Compound (QUAT)

QUATs are often used for routine cleaning on board airplanes and in the food industry [5]. They possess the active ingredient benzalkonium chloride and are very widely used [6].

QUATs are characterized as low level disinfectants, killing most vegetative bacteria and some fungi and viruses in less than 10 minutes [2]. Further, QUATS are considered to have a low toxicity rating and are generally stable. QUATS are associated with bacterial resistance and toxicity to aquatic life [7, 8].

3.1.5 Sodium Hypochlorite

Another disinfectant used on airplanes is sodium hypochlorite, in varying concentrations. The World Health Organization recommends sodium hypochlorite diluted to strength of 100 mg/L [9]. The chlorine solution is an oxidizing agent, bleaching agent, and disinfectant [10]. Per the Spaulding Table for Disinfection, a guideline classifying disinfectants and surfaces to be disinfected, sodium hypochlorite should only be used on hard surfaces on intermediate and low level critical items [2]. These are items that may contact mucous membranes or non intact skin (semicritical) or items that come in contact with intact skin but not mucous membranes (non-critical) [2]. Hypochlorite solutions are classified as irritants and corrosive, it is necessary to take precautions when using concentrated hypochlorite in the form of bleach as a disinfectant [3].

3.1.6 Hypochlorous Acid

When sodium hypochlorite is added to water, hypochlorous acid is formed [11]. In water or liquids, the amount of hypochlorous acid (HOCl) in combination with hypochlorite ions (ClO^-) is referred to as free chlorine [11]. There is a balance between HOCl and ClO^- that relies on the pH and temperature of the liquid [11]. The more hypochlorous acid that is present, the more effective the liquid is as a disinfectant, since HOCl is the more reactive and bacteriocidal of the two [12]. Hypochlorous acid is nontoxic to humans, making using it during a quick flight turnaround time less problematic [12].

Though hypochlorous acid is valued for its bacteriocidal properties while remaining non-toxic, it has a limited shelf life [12]. Hypochlorous acid solutions can convert to hypochlorite, the chlorine derivative used in bleach, if the pH changes from the desired range of 3.5-5 [13]. If the pH raises to 5.5, the hypochlorite ion is formed. At a pH of greater than 9.5, 100% hypochlorite is obtained [13]. This is detrimental because hypochlorite derivatives can be toxic to humans [10, 14]. If solutions containing hypochlorous acid are exposed to the light or sunlight in the presence of catalysts, the HOCl will decompose to hydrochloric acid and oxygen [15]. Hydrochloric acid is a highly corrosive and strong acid.

3.1.7 Hypochlorous Acid Mechanism of Action

Hypochlorous acid (HOCl) is thought to be utilized by phagocytes to inhibit pathogens [12, 16]. It has many different abilities that all work towards its bacteriostatic ability. The disinfectant produces reactive oxygen species that are similar to those produced by hydrogen peroxide [16]. HOCl has also been shown to inhibit DNA and protein synthesis [17]. In concentrations of 0.1μM, HOCl was found to be bacteriocidal to *E. coli*, killing $1.5x10^7$ in less than 5 minutes [17]. 19.1 μM of HOCl is equivalent to 1 ppm, indicating that a very small concentration of HOCl can kill a large amount of bacteria within a relatively short amount of time [18]. HOCl has been found to inactivate enzymes and electron transport systems as well as oxidize nucleotides [12]. All of these abilities could contribute to the inhibition of cell division and growth. Additionally, hypochlorous acid can disrupt cell membranes. This could potentially lead to cell lysis and death [12].

3.1.8 Nature Unleased Anolyte

A hypochlorous acid derivative sprayed from a mister under controlled conditions was applied to develop a disinfection model. The derivative used for the purpose of this study was

provided by Nature Unleased™ and is called Anolyte. Anolyte is electrolyzed salt water that is used to produce hypochlorous acid. The resulting disinfectant contains 500 ppm of free available chlorine and, in addition to being an Environmental Protection Agency registered product, is approved by the Food and Drug Administration as a medical device. The concentration of hypochlorous acid in the product is .046%. Nature Unleased™ Anolyte is recommended for disinfection on hard, non-porous surfaces. The disinfectant poses no environmental concern and is non-toxic. The shelf life of Anolyte, however, is 30 days and the product must be stored in a cool, dark place.

3.1.9 The Ideal Disinfectant

In addition to the two disinfectants recommended by the WHO, further research has been conducted on many other disinfectants to determine effectiveness on board airplanes [19]. However, airplane cabins pose an additional challenge when selecting a disinfectant. As previously mentioned, rapid flight turnaround times means that disinfection of cabins must be fast-and no residues or scents should remain after disinfection is complete. Furthermore, the disinfectants used should not be toxic or corrosive. The plethora of people flying on airplanes each day possess a variety of known allergies, meaning the disinfectants used on planes must be non-irritating. With a variety of materials on an airplane, disinfectants that degrade or corrode surfaces are not ideal. Airplanes do not have much storage space, so the disinfectant needs to packaged in a way that is ideal for airplane storage [19]. The ideal disinfectant also should be easy to prepare and use. Additionally, disinfectants that could be incorporated into a coating would allow for extended bacterial killing and a lower frequency of application.

3.2 Pathogens used for Nature Unleased™ Anolyte Studies

In addition to *Streptococcus pyogenes* strains with the M1 and M5 proteins, *Escherichia coli* O157:H7 and Methicillin Resistant *Staphylococcus aureus* strains were used. *E. coli* O157:H7 was selected due to its pathogenic nature as a cause of foodborne illnesses and outbreaks. MRSA was selected due to its ability to cause skin infections. Additionally, both organisms are commonly transmitted by contact and surface transmission.

3.3 Methicillin Resistant *S. aureus* Background

Staphylococcus aureus, a Gram positive coccus notorious for causing painful skin infections, was discovered in the 1880s [20]. The organism has a high affinity for bloodstream infections and is the primary cause of hospital acquired pneumonia [21]. The invention of penicillin by Alexander Fleming in 1928 significantly advanced the treatment of *S. aureus* infections [22]. However, less than 20 years later, the organism had already largely developed resistance to the drug [20]. By the 1960's, 80% of infections were completely resistant to penicillin [23]. To combat *S. aureus* resistance to penicillin, a penicillinase-resistant penicillin, methicillin, was introduced in the 1960s. Within a remarkably brief period of time, *S. aureus* strains resistant to methicillin (called MRSA) appeared with the first documented case of MRSA in the United States reported in 1968. The strain is resistant to all beta lactams and is associated with high mortality and morbidity [23]. Today, two in 100 people are carriers of MRSA and there are over 80,000 cases per year [24].

3.3.1 MRSA Classifications

MRSA infections are now recognized as hospital and community acquired, making MRSA a household name. MRSA has recently encouraged awareness by infecting people with no risk factors for infection [25]. In fact, clones of community isolated MRSA have been isolated from the skin abscesses of football players. The pathogen is largely grouped as either hospital

acquired (HA-MRSA) or community acquired (CA-MRSA) [21]. CA-MRSA typically causes milder skin infections, but can also infect the lungs [26]. HA-MRSA, on the other hand, causes surgical site and blood stream infections that are often fatal. HA-MRSA is found in immunocompromised people or those with recent hospitalization, surgery, or stays in a long term care facility. In contrast, CA-MRSA can infect anyone and most commonly affects young, healthy people. Further, CA-MRSA is susceptible to most non beta-lactam antibiotics while HA-MRSA is commonly multidrug resistant [26].

3.3.2 MRSA Transmission

MRSA is transmitted through direct skin to skin contact or contact with items that an infected person has interacted with [27, 28]. MRSA is most likely to be transmitted when there is crowding, frequent skin to skin contact, cuts or abrasions, contaminated items, or a lack of cleanliness involved. Examples of environments that meet these criteria are schools, daycare facilities, correctional facilities, dormitories, military barracks, and airplane cabins. Numerous healthy people are unknowingly carriers of MRSA on their skin, helping to proliferate the organism. The only time some of these people may become aware of their colonization is if they develop a cut or abrasion that becomes infected with MRSA. Some carriers develop minor skin infections such as boils regularly. Though the nose is most commonly colonized, sites such as the groin and intestinal tract can also be colonized in people who are carriers of the pathogen [27, 28].

3.3.3 *S. aureus* Pathogenesis

S. aureus possesses an arsenal of virulence factors used to cause disease [28]. The organism uses both secreted and structural factors to establish an infection and disseminate [28]. Additionally, some virulence factors may play multiple roles while others are known to have the

same function. This allows strains with only selective virulence factors to still have the capability of successful infection.

3.3.4 Establishing Infection

The surface proteins of *S. aureus*, called microbial surface components recognizing adhesive matrix or MSCRAMMS, are responsible for the organism's attachment to host tissues [28]. The molecules bind to fibrinogen, collagen, or fibronectin, playing a role in the initiation of bone infections, endovascular infections, and infections of a prosthetic device. Some MSCRAMMS may be more prone to cause specific types of infection based on their attachment preferences. For this reason, *S. aureus* may have multiple different types of MSCRAMMS for different infections.

After attachment, *S. aureus* must evade the host immune response. The organism does this using many different techniques [28]. One ability to evade the host response comes from the pathogen's tendency to form biofilms [28, 29]. *S. aureus* can also form small colony variants (SCVs) that allow the organism to remain in host cells without recognition until reversion and recurrent infection. Like many other successful pathogens, *S. aureus* has a capsule that helps it evade phagocytosis.

A key trait of *S. aureus* is the organism's capability to disseminate throughout the host, past the initial point of infection. This is achieved through the production of enzymes like proteases and lipases that destroy host tissues. *S. aureus* can further metastasize with production of of alpha toxins, superantigens, and other toxins like exfoliative toxins. These products can cause septic shock and toxin caused illnesses such as toxic shock syndrome, food poisoning, and scalded skin syndrome.

The success of *S. aureus* as a pathogen can also be attributed to the organism's regulation of virulence factors. For example, MSCRAMM expression occurs during log phases of growth. This is due to MSCRAMMs function in adherence and attachment being needed early on in the life cycle of the organism. Once *S. aureus* is inside the host, toxins and other secreted proteins are produced. This occurs when the organism is in stationary phase, allowing the pathogen to spread to new sites. *S. aureus* has developed quorum sensing that is used while in biofilm to regulate the production of virulence factors and allow communication with the other cells of the biofilm. This quorum sensing, or cell-to-cell communication, is enabled by the accessory gene regulator system (agr) in *S. aureus*. This system aids in the aforementioned increase in secreted virulence factor expression during stationary phase [28, 29].

3.3.5 Penicillin and Methicillin (Meticillin) Resistance

Genetically, MRSA has been extensively characterized. A requirement of the strain is the possession of the *mec* gene, which results in *mecA*, encoding penicillin binding protein 2a (PBP2a) that confers resistance to methicillin (now called meticillin) [25, 30]. *S. aureus* commonly possesses 4 PBPs, all of which are sensitive to beta lactam antibiotics [31]. Like PBP2a, these 4 PBPs function in peptidoglycan biosynthesis. Specifically, these proteins work to regulate and execute cross linking, also called transpeptidation of glycine residues located between the L-lysine of one peptide to the D-alanine residue of a second peptide [31]. This helps attach glycan chains to form strong layers of peptidoglycan that provide structure for the cell [31, 32, 33].

Beta lactam antibiotics include penicillin and meticillin [34]. Beta lactams kill *S. aureus* by binding to transpeptidases and preventing transpeptidation to form PTG. Without this, the cell will eventually die [35]. Strains became resistant to penicillin with the production of

penicillinase, which is encoded for on the same transposable element that includes many antimicrobial resistance genes [23]. The beta lactamase penicillinase hydrolyzes the beta lactam ring of the antimicrobial, rendering it useless.

MRSA strains possess the penicillin binding protein PBP2a, which has a very low affinity for beta lactam antibiotics. In fact, the protein's active site blocks beta lactams from binding, allowing transpeptidation to continue. The possession of the *mec* gene and subsequent production of PBP2a renders the *S. aureus* strain resistant to every beta lactam. Quinolones and vancomycin have been used to treat these strains, however, resistance has developed to both drugs. The drug of choice to treat MRSA today is vancomycin. For vancomycin resistant strains, new drugs such as linezolids and preventative isolation of infected people are the current treatment regimes [23].

3.4 *Escherichia coli*

E. coli is a gram negative, motile, facultative, short to medium length rod that was first discovered in 1885 [36, 37]. *E. coli* strains cause many different diseases, including diarrhea, septicemia, pneumonia, meningitis, dysentery, and bladder and kidney infections [36, 37]. However, each strain is responsible for only one condition due to the virulence traits acquired by that particular strain [34]. In fact, many strains of *E. coli* are not virulent and the organism is frequently used as a model for research in bacteriology. To differentiate between pathogenic, non-pathogenic, and specific disease causing strains, groupings were put into place to further characterize the organism.

3.4.1 Classification

E. coli was first grouped based on bacterial surface antigens, or serotyping/serogrouping [34]. The O antigen of lipopolysaccharide (LPS) and the H antigen which stands for hauch (the

German word for flagella) were used to identify the serogroup and serotype, respectively. If the strain has a capsule, the capsular antigen, or K, is also used for designation. There are more than 700 characterized serotypes of *E. coli*, making the organism very diverse. Due to the repertoire of different diseases caused by the organism, virotyping, or the classification based on virulence traits possessed by the strain is also utilized when designating *E. coli* strains. Virotypes are based on the method of bacterial attachment to host cells, production of toxins, effect on host cells, and invasiveness of the organism. The six different diarrheal virotypes belonging to *E. coli* are exterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrrhagic *E. coli* (EHEC), enteroinvasive *E. coi* (EIEC), enteroaggregtive *E. coli* (EAEC), and diffusely adhering *E. coli* (DAEC). Some strains of the organism can be associated with more than one class.

Enterohermorrhagic *E. coli* is responsible for many outbreaks and can result in hemolytic uremic syndrome (WHO). EHEC is also very prominent in developed countries like the United States and Canada. EHEC binds in a patchy like fashion to host cells, producing a dramatic response. Around 10% of EHEC patients will develop hemolytic uremic syndrome. The most well known and virulent member of EHEC *E. coli* is *E. coli* O157:H7 [34].

3.5 *E. coli* O157:H7 Overview

E. coli O157:H7 was first recognized as a human pathogen in 1982 following multiple outbreaks involving the organism [36]. Both outbreaks involved undercooked beef patties from the same fast food restaurant chain. Overall, there were 47 cases and 33 hospitalizations from the outbreak. *E. coli* O157:H7 was also linked in 1983 to children with hemolytic uremic syndrome [36]. Today, *E. coli* O157:H7 is responsible for around 95,000 cases in the United State per year [38]. The organism is a fecal pathogen, with cattle as the primary reservoir, that is linked to gastrointestinal illness outbreaks and high morbidity and mortality around the world. Cattle

asymptomatically carry *E. coli* O157:H7 and shed it in their feces for periods of a month or longer [39]. For this reason, contaminated beef products are the most common source of *E. coli* infection [39]. The virulence factors of *E. coli* O157:H7 set it apart from the other strains as a more virulent pathogen.

3.6 *E. coli* O157:H7 Virulence Factors

3.6.1 Shiga Toxins

EHEC strains, including *E. coli* O157:H7, possess shiga toxins (stxs) [39]. These toxins are divided into Stx1 and Stx2, with Stx1 being almost identical to the Stx belonging to *Shigella dysenteriae*. One or both toxins can be expressed by virulent *E. col* O157:H7 strains. Stx2 is associated with HUS in humans and is considered more toxic than Stx1. Shiga toxins are encoded by bacteriophage and possess a conserved structure of one enzymatically functioning A unit and 5 B units that act to bind specific host receptors. After the B subunits bind, the A subunit is internalized and inhibits protein synthesis by removing an adenine residue from the 60S ribosomal subunit of 28S rRNA [39].

3.6.2 Plasmid O157

Every strain of *E. coli* O157:H7 isolated from human infection has possessed the nonconjugative, F-like plasmid pO157 [39]. Located on the plasmid are many potential virulence factors. The first, Hemolysin (Ehx), is responsible for lysing human erythrocytes and destroying bovine leukocytes [40]. However, it has no activity against human leukocytes. The hemolysin operon *ehxCABD* encodes for the virulence factor [39, 40]. It has a G+C content percentage different from other genetic contents possessed by the strain, indicating that it may have been acquired by *E. coli* O157:H7 over time.

Plasmid O157 also encodes the genes for Catalase-Peroxidase (KatP) enzyme, which acts to help *E. coli* O157:H7 disseminate by reducing oxidative stress. Open reading frames correlating to Type II secretion systems are also located on the plasmid. Genes for metalloproteases, serine proteases, and adherence contributing proteins like ToxB are also found on the plasmid [39, 40].

3.6.3 Identification of *E. coli* O157:H7

E. coli are fermenters of lactose and sorbitol [41]. However, *E. coli* O157:H7 does not ferment sorbitol, making Sorbitol MacConkey Agar (SMAC) an appropriate media for differentiation between *E. coli* O157:H7 and non-O157:H7. MacConkey agar (MAC) contains lactose, so fermenting non-O157:H7 and *E. coli* O157:H7 are indistinguishable. On SMAC, however, *E. coli* O157:H7 will appear colorless due to its inability to ferment sorbitol. Non-*E. coli* O157:H7 appears pink on SMAC, differentiating the two [41]. To further identify *E. coli* O157:H7, O157 antiserum or latex reagents can be used [42]. Identification of the H7 flagellar antigen is the last step for confirmation. If isolates are negative for the H7 flagellar antigen, testing for the presence of Shiga like toxins should be conducted [42].

Summary

The lack of an adequate cleaning routine, documented outbreaks, and high risk factors for transmission on board of airplanes render a need for the characterization of disinfectants and a disinfection routine to be used in airplane cabins. Hypochlorous acid is an ideal disinfectant for airplane cabins due to its high efficacy against many pathogens, non-toxicity to humans, and ease of use. Three very prominent pathogens: *S. pyogenes*, MRSA, and *E. coli* O157:H7 are susceptible to hypochlorous acid and were used in this study. MRSA and *E. coli* O157:H7 have established themselves as the predominant pathogenic strain of their genus and species through the acquisition and regulation of virulence factors. The characterization of Anolyte's effects on

the three pathogens could provide insight into the development of a disinfection scheme for airplanes.

Tables and Figures

Symbols: ✓ Standard + On request
A: Stopover times under 60 minutes
B: Stopover times over 60 minutes
C: Overnight

Area	Services	A	B	C	Remarks
Flight deck	Empty waste boxes and ashtrays	✓	✓	✓	
	Clean crew tables and glass holders	+	✓	✓	
	Clean stowage areas and racks	+	✓	✓	B: As required
	Wipe seats	+	✓	✓	Remove stains
	Clean floor / Vacuum carpet	+	+	✓	
	Clean flight deck windows inside	+	+	✓	
	Clean door and walls	+	+	✓	
Cabin	Dispose of waste from closets	✓	✓	✓	
	Dispose of litter and newspapers	✓	✓	✓	
	Dispose of waste in seat pockets		✓	✓	
	Collect and restow pillows and blankets (first, business class)	✓	✓	✓	Remove if soiled
	Fold and restow blankets in overhead bins	✓	✓		Remove if soiled
	Restow pillows in overhead bins	✓	✓		Remove if soiled

Area	Services	A	B	C	Remarks
	Empty ashtrays		✓	✓	
	Clean tray tables and armrests	+	+	✓	
	Clean cabin crew seat tables	+	+	✓	
	Clean interphone surfaces	+	✓	✓	
	Clean cabin windows inside			✓	
	Vacuum passenger and cabin crew cloth-covered seats		+	✓	Remove stains
	Wipe passenger and cabin crew leather-covered seats		+	✓	Remove stains
	Dispose of waste in overhead bins and wipe		+	✓	
	Clean overhead bins outside and latch handle surfaces	+	+	✓	
	Clean PVC floors			✓	A: As required
	Vacuum carpet		+	✓	A: As required
	Empty and clean ashtrays			✓	
	Vacuum ashtray holders			✓	
	Collect and replace blankets			✓	
	Collect and replace pillows			✓	
	Collect and replace headrest covers			✓	
	Clean in-seat monitors			✓	
	Clean passenger seat/service control unit panels	+	+	✓	
	Remove passenger seat cushions and vacuum			✓	
	Remove stains from carpets			✓	
	Clean seat rails, cabin fixtures, air inlets, ceiling, sidewalls, closets, doors, service panels and magazine racks			✓	
Galleys	Empty waste bins and insert waste bags	✓	✓	✓	
	Clean doors, latches, ceiling, ventilation grids	+	+	✓	
	Clean sinks, faucets and working surfaces	+	✓	✓	
	Clean retractable tables	+	✓	✓	
	Clean ovens inside and outside	+	+	✓	
	Clean service trolleys	+	✓	✓	
	Clean PVC floors	+	+	✓	
Lavatories	Empty waste bins and insert waste bags	✓	✓	✓	

Area	Services	A	B	C	Remarks
	Clean toilet bowl and seat	✓	✓	✓	
	Clean basin, faucets and surfaces	✓	✓	✓	
	Clean mirror	✓	✓	✓	
	Clean change table	✓	✓	✓	
	Clean wall surfaces and interior and exterior door handles and locks	✓	✓	✓	
	Clean PVC floors	✓	✓	✓	
	Replenish soap dispenser	+	✓	✓	
	Replenish toiletry items	+	✓	✓	
Crew rest areas	Dispose of waste from closets		✓	✓	
	Dispose of litter and newspapers		✓	✓	
	Remove sheets, pillows and blankets from each sleeping berth		✓	✓	This step followed by next two in sequence
	Clean surfaces within each sleeping berth		✓	✓	
	Place clean sheets on mattresses and clean pillows and blankets in each sleeping berth		✓	✓	
	Clean controls (for lights and ventilation, etc.) and interphone surfaces		✓	✓	
	Empty ashtrays		✓		
	Vacuum carpet				A: As required
	Clean any cabin crew seat tables		✓	✓	
	Clean any cabin windows inside		✓	✓	

Table 4. Guidelines for airplane cabin cleaning. Taken from:

http://www.who.int/ihr/ports_airports/guide_hygiene_sanitation_aviation_3_edition_wcov.pdf

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Chapter 4

Disinfection of Airplane Cabin Coupons Mounted with Pathogens

Abstract

To provide a solution for the results from the survivability and transmissibility studies, we investigated the use of a hypochlorous acid product (Nature Unleashed™ Anolyte) for disinfection. The solution was misted onto coupons separately mounted with *S. pyogenes*, *E. coli* O157:H7, and Methicillin Resistant *S. aureus* for 10 minutes. Coupons were incubated with a neutralizer to determine survival rates. We concluded that the mister containing anolyte was the most bactericidal when placed 9 ft. away from the samples. In addition, MRSA was killed by the disinfectant at a concentration of 1 ppm and *E. coli* O157:H7 at 2 ppm after 10 minutes of exposure. *S. pyogenes* was killed by a 6 ppm concentration of Anolyte.

Introduction

The alarming lack of a uniform disinfection protocol, as depicted in **Table 1**, indicates the great need for investigation of an ideal disinfection procedure to be used on board of airplanes. With numerous factors such as quick flight turnaround times, potential passenger exposure to the disinfectant, and multiple surface types on board the plane needing disinfection, it is challenging to identify a disinfectant that meets the extensive criteria. Hypochlorous acid, a disinfectant that, when in solution, meets most of the specifications, was characterized further in

this study. Additionally, well established pathogens *S. pyogenes*, MRSA, and *E. coli* O157:H7 were used to document the efficacy of the disinfectant. Since flight turnaround times pose the problem that the wiping of all surfaces with disinfectant might not be possible, the possibility of disinfection using a misting device to mist disinfectant onto the surfaces was explored.

Materials and Methods

We used *E. coli* O157:H7 (ATCC® 43894™), an ATCC isolate that was taken from a patient with hemorrhagic colitis [1]. Additionally, we used a community acquired Methicillin Resistant *S. aureus* strain also acquired from ATCC (ATCC® BAA1707™) that was originally isolated from an outbreak in 1988 [2]. Both strains were initially stocked in 20% (v/v) glycerol and maintained at -80°C. The strains were resuscitated separately in 10mL of Tryptic Soy Broth (TSB) and incubated until mid log phase at 37°C, with shaking. A master plate of each strain was made after the strains were confirmed by using Sorbitol MacConkey plates for *E. coli* O157:H7 and Staph 110 plates for MRSA.

Overnight cultures of *S. pyogenes*, *E. coli* O157:H7, and MRSA were grown in THB and BactoTM Tryptic Soy Broth (TSB, TSB; Beckton Dickinson and Co., Sparks, MD), respectively, then spun down to remove growth medium. The pellets were re-suspended in PBS. OD_{600} values were obtained and adjusted to 1.0 for GAS, .6 for MRSA, and .5 for *E. coli* O157:H7. The *S. pyogenes* tubes were sonicated for 4 10-second bursts to break the characteristic chain-like arrangement of the organism. The CFU/mL cell concentration of $1x10^8$ was confirmed through serial dilution and plating for all organisms. A constant 10 µL of bacteria was added to a mixture of Nature Unleashed® Anolyte disinfectant and sterile water correlating to 50, 40, 30, 20, 9, 8, 7, 6, 5, 4, 3, 2, and 1 parts per million (ppm) of Anolyte. The bacteria were exposed to the solution for 10 minutes then immediately plated onto Tryptic Soy Agar (TSA, Becton, Dickinson and

Company, Sparks, MD) or SBA plates and allowed to incubate for 24 and 48 hours at 37°C. Colony forming units/mL were then assessed by plate counts to determine the minimum bactericidal and inhibitory concentrations (MBC).

After MBC numbers were determined, an experiment was conducted involving the pathogens, anolyte solution, and a mister (Hurricane Model 2790/2792, Curtis Dyna Fog, Westfield IN). Anolyte, at a concentration of 500 ppm, was loaded into the mister and the mister was placed 12 inches above the coupon surface. Once loaded with 25 µL of 10^8 CFU/mL bacteria and allowed to dry, coupons were placed 5, 9, and 12 feet from the mister. 3 coupons per pathogen in a sterile petri dish were placed at these distances in the order shown in **Figure 12**.

The mister was turned on for 10 minutes as Anolyte was misted onto the coupons. After this exposure, the coupons were aseptically transferred to individual conical tubes containing 5mL of the appropriate growth media and sodium thiosulfate, a chlorine neutralizer. The conical tubes were incubated at 37°C and monitored every 24 hours for 48 hours. Positive tubes were discerned by the presence of turbidity. All positive tubes were struck onto agar plates to eliminate the possibility of contamination by observation of colony morphology. Two separate overnight cultures were grown per genera of bacteria. Three coupons per culture were exposed to the mister, giving a total of 6 coupons per distance per genera. The experiment was repeated once. This experiment was structured based on the standard operating procedure for the use-dilution method published by the AOAC and EPA [3].

Results

After incubation for 24, then 48 hours, the bacterial cultures were checked for the presence of turbidity. Within 24 hours at a 5 ft. distance from the mister, 4 of the 12 coupons mounted with *E. coli* O157:H7 that were placed into THB and Sodium thiosulfate neutralizer

possessed turbidity while 2 of the 12 MRSA and 0 of the 12 *S. pyogenes* containing the M1 strain coupons showed turbidity. At 48 hours of incubation of the coupons mounted with a pathogen and placed at a 5 ft. distance from the mister, 4 of the 12 *E. coli* O157:H7 samples showed turbidity, with 5 of 12 MRSA and 1 of 12 GAS showing growth.

The results of the study are shown in **Table 4**. When placed at a 9 ft. distance from the mister, coupons placed in THB and sodium thiosulfate monitored at 24 hrs. showed no visible growth for any pathogen. When assessed at 48 hours, turbidity was only noticed for *S. pyogenes*. At a 12 ft. distance, *E. coli* O157:H7 possessed turbidity in every tube but one for both the 24 and 48 hour checkpoints. MRSA and GAS, however, showed no growth. Every tube possessing turbidity was struck onto the appropriate growth media and identified to ensure no contamination. All organisms were ensured to be the correct pathogen being tested, and no contaminants were discovered.

The placement of each pathogen beside each another as shown below provided a unique opportunity for to compare growth of the organism in order to speculate on the range out from the mister that the appropriate amount of solution for disinfection could be delivered. *S. pyogenes* Run #1 at 9ft. *S. pyogenes* (A) and *E. coli* O157:H7 (B), both located the furthest from the mister, had more growth than their *S. pyogenes* (B) and *E. coli* O157:H7 (A) counterparts. In fact, almost all of the growth observed from GAS came from the *S.* pyogenes (A) coupons. The same proved true for *E. coli* O157:H7 (A) vs *E. coli* O157:H7 (B).

Discussion

Overall, the bacteria sampled had the least growth at a 9 ft. distance from the mister. A pattern of growth can be seen in the *E. coli* O 157:H7 coupons, with multiple coupons located 5 feet from the mister possessing the pathogen after Anolyte was misted and almost every coupon

at 12 feet showing turbidity upon incubation. Furthermore, MRSA and GAS mounted coupons showed turbidity when located 5 feet from the mister, indicating that a 5 feet distance was too close to the mister for adequate killing using Nature Unleashed™ Anolyte with an exposure time of 10 minutes.

All pathogens located 9 feet from the mister lacked visible turbidity at 24 hours post incubation. This indicates that, of the 3 lengths from the mister sampled, 9 feet is the ideal range to place the device from the desired surface to disinfect when using Nature Unleashed™Anolyte solution at 500 ppm. 2 coupons of GAS placed 9 feet from the mister did indicate turbidity at 48 hours, however. This is an interesting find considering pure hypochlorous acid has been successful when used in conjuncture with other treatments for *S. pyogenes* caused necrotizing fasciitis [4]. The turbidity was only observed in the first run, and was only located in 2 tubes out of 12 after 48 hours of incubation. The growth could be attributed to the ability of GAS to repair enough damage caused by the hypochlorous acid to survive. Since the growth was not observed when the experiment was repeated, the ability of typical GAS to regularly continue to grow after exposure to Nature Unleashed™ Anolyte at a 9 ft. distance is unlikely. Further, the lack of turbidity in almost every tube of GAS after Nature Unleashed™ Anolyte exposure when compared to positive control of GAS mounted to the coupons then incubated for 24 and 48 hours in THB with sodium thiosulfate indicates that the hypochlorous acid solution is damaging to the organism.

All three organisms showed some lag of regrowth at 48 hours, meaning that at 24 hours, a tube had no visible turbidity but was turbid at 48 hours. Lag of regrowth is a result of the ability of an organism to repair itself to survive or an indication of the survival of a mutant population resistant to the disinfectant [5]. Typically, this phenomenon is most frequently observed in

stationary phase bacteria are likely stressed and prone to mutations [6]. Since we attempted to mimic conditions most commonly found in nature, all bacterial cultures were taken when they were in late log to early stationary phase growth. This could have contributed to the lag of regrowth of the pathogens observed. Further, the lack of turbidity in a tube does not indicate that there is no growth in the tube. This is exemplified in the tubes that showed turbidity at 48 hours but not at 24.

GAS had significantly less growth than both MRSA and *E. coli* O157:H7. This could be for potentially many reasons. Firstly, MRSA and *E. coli* O157:H7 are both facultative anaerobes, able to happily survive in the presence of oxygen. Conversely, *S. pyogenes* prefers 10% Carbon dioxide for growth and there is only .04% in the atmosphere [7, 8]. This could contribute to the reduced survival of *S. pyogenes*. Also, *S. pyogenes* lacks catalase enzymes while MRSA and *E. coli* O157:H7 possess them [9]. Since HOCl is an oxidizing agent, these enzymes could help protect MRSA and *E. coli* O157:H7 [9]. The lack of catalase could be detrimental for *S. pyogenes* when exposed to HOCl.

MRSA samples had less growth than *E. coli* O157:H7 overall. This could be due to the slightly lower bactericidal concentration of Nature Unleashed™ Anolyte for MRSA (MBC=1 ppm) versus *E. coli* O157:H7 (MBC=2 ppm). Also, the organism may not respond to oxidative stress produced by HOCl as well as *E. coli* does. Other studies have shown *S. aureus* to respond to HOCl induced stress with the utilization of MgrA, a protein used to prevent DNA from experiencing oxidative damage and the increased production of alkaline phosphatase [10]. Alkaline phosphatase is used by the organism to induce ς^B , a general stress response alternative sigma factor of *S. aureus* [10, 11, 12]. Interestingly, some other studies have shown that *S. aureus* is more resistant to HOCl than *E. coli* when a greater log reduction of *E. coli* was

observed after exposure to the disinfectant [10, 13]. In our studies, the location of MRSA directly in front of the mister and *E. coli* O157:H7 placed slightly to the side of the mister could have influenced results. Since MRSA was located directly in front of the mister, there is a possibility it was misted with a larger quantity of Anolyte solution than *E. coli* O157:H7 mounted coupons. Evidence for this observation can be further observed with the growth of *E. coli* O157:H7 (A) versus *E. coli* O157:H7 (B). (A) was placed closer to the mister than (B), as observed in **Figure 12**. Coincidentally, (A) showed markedly less growth than (B). In the (A) tubes, 5 of 18 total tubes showed turbidity at 48 hours and 10 of 18 total (B) tubes had observable growth. The observation of less growth in the coupons containing the *E. coli* O157:H7 tubes closest to the mister was confirmed in both runs of the experiment.

E. coli O157:H7 showed the most turbidity in the mister experiments. The ability of the organism to combat the effects of hypochlorous acid is likely due to its possession of the σ^s factor that is encoded by the *rpoS* gene. Previous studies show that the pathogen reacts to HOCl with the same mechanisms that it uses to combat H_2O_2. This is likely because both produce the same reactive oxygen species, but react differently with molecules [9]. The σ^s factor regulates many catalases and proteins that are imperative to the organism's the resistance to hydrogen peroxide. In addition, the ability of *E. coli* to perform recombination repair and its possession of the *oxyR* regulon also prove important HOCl resistance [9].

Though there was still some survival of the organisms in the mister experiment, it is important to note the efficacy of Nature Unleashed™ Anolyte when it is directly applied to the bacteria. The minimum bacteriocidal concentrations (MBCs) determined for each organism were complete killing of MRSA at 1 ppm, *E. coli* 0157:H7 at 2 ppm and an MBC 90 for *S. pyogenes* at 4 ppm. 460 ppm of hypochlorous acid is in a bottle of Anolyte disinfectant per the labeling.

We concluded from this information that though Anolyte is effective at killing all three pathogens, the mister used in the study did not adequately deliver enough Anolyte to the coupons at 5 and 12 feet. However, at all distances from the mister measured, there was still significant inhibition of bacterial growth. The percentages of growth after exposure to Anolyte are depicted in **Table 6**.

Conclusion

In this study, we documented the ability of Nature Unleashed™ Anolyte to kill Methicllin Resistant *S. aureus, E. coli* O157:H7 and *S. pyogenes* strains by determining the Minimum Bacteriocidal concentration for each. We also used a mister device loaded with the Anolyte disinfectant to spray the disinfectant onto coupons pre-mounted with one of the three pathogens to determine the ability of the mister to disperse the disinfectant. We concluded that Nature Unleashed™ Anolyte has the ability to kill all three pathogens at a low concentration and that the mister is the most effective when placed 9 ft. away from the surface to be disinfected. Though the mister did prove to be effective, this device alone is not feasible to be used in an airplane cabin due to the 9ft. restrictions and further research is needed.

Figures and Tables

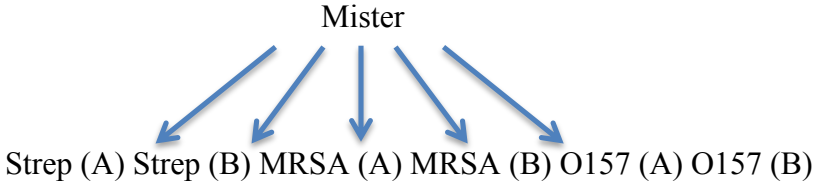

Figure 12. The order of coupons for the mister experiment.

Distance and Time		Pathogen Used		
		O157:H7	MRSA	GAS
5 ft.	24 hr.	4 of 12	2 of 12	0 of 12
	48 hr.	4 of 12	5 of 12	1 of 12
9ft.	24 hr.	0 of 12	0 of 12	0 of 12
	48 hr.	0 of 12	0 of 12	2 of 12
12ft.	24 hr.	11 of 12	0 of 12	0 of 12
	48 hr.	11 of 12	0 of 12	0 of 12

Table 5. The y axis headings describe the distance from the mister (i.e. 5, 9, 12 ft.) and the hour at which turbidity was assessed (24 vs. 48). The x axis describes the pathogen used, *E. coli* O157:H7, Methicillin resistant *S. aureus*, or *S. pyogenes* strain possessing the M1 protein.

Distance and Time		Pathogen Used		
		O157:H7	MRSA	GAS
5ft.	24 hr.	33%	16%	0%
	48 hr.	33%	42%	8%
9ft.	24 hr.	0%	0%	0%
	48 hr.	0%	0%	16%
12ft.	24 hr.	92%	0%	0%
	48 hr.	92%	0%	0%

Table 6. Percentage of pathogen surviving after 10 min. exposure time to mister loaded with Anolyte. Survival is based on the presence of turbidity in a tube containing growth medium, neutralizer, and coupon with bacteria after 24 or 48 hour incubation periods.

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