Survival and Transmission of Selected Pathogens on Airplane Cabin Surfaces and Selection of Phages Specific for *Campylobacter jejuni*

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
May 3, 2014

Keywords: survival, transmission, pathogens,
surfaces, aircraft cabin

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Abstract

We determined the limits of survival and transmission of *Escherichia coli* O157:H7 and methicillin-resistant *Staphylococcus aureus (*MRSA) on common surfaces found in the cabin of a civil aircraft. The two pathogens were suspended in phosphate buffered saline (PBS), simulated sweat and saliva. A concentration of 10^5 cells/mL of the three suspensions was inoculated onto coupons (1 x 1 cm^2) of the polyurethane armrest, metal toilet handle, plastic tray, plastic window shade, leather seat and pocket cloth. The coupons were exposed to typical airplane cabin conditions of 24° C and 18% humidity. Direct sonication was employed to detach the bacteria right after drying and intermittently every 24 hours. Real-time quantitative PCR (qPCR) in combination with propidium monoazide (qPCR-PMA) and plate counts were performed to monitor the persistence of *E. coli* O157:H7 in viable but non-culturable (VBNC) state. MRSA survival was quantified by plate counts. A surrogate pigskin contact model was employed to establish the transfer rates of the two pathogens from the contaminated surfaces to skin over time. The general linear mixed effects model with repeated measures was used to analyze the data. The qPCR-PMA method eliminated the amplification from dead cells and was significantly more sensitive than the plate counts to establish the limits of *E. coli* O157:H7 survival ($p < 0.05$).

E. coli O157:H7 persisted the longest on armrest, (96h) followed by tray table (72h) and toilet handle (48h) and remained viable longer in PBS, followed by sweat and saliva ($p < 0.05$). Recovery rates for MRSA decreased over time but more rapidly for non-porous compared to porous fomites ($p < 0.05$). The organism was culturable significantly longer in PBS, followed by saliva and then sweat suspension ($p < 0.05$). The pigskin model indicates that transmission rates for both pathogens are significantly higher on non-porous materials when compared to porous surfaces ($p < 0.05$). Our experiments indicate that *E. coli* O157:H7 and MRSA can survive for several days on surfaces with different physicochemical properties in the presence and absence of organic matter with different pH. To our knowledge, the current study is the first attempt to elucidate the relative risk of pathogen transmission from contaminated surfaces to the hands of the passengers during a commercial airplane flight.

The last chapter of this dissertation describes the trials performed for the isolation of phage displayed peptides with specificity for *Campylobacter jejuni*. The isolated clone 17 with displayed peptide QGAQARSGTPVQ showed a high degree of binding to *Campylobacter jejuni*, but also cross-reacted with two other members of the genus *Campylobacter*, *Campylobacter coli* and *Campylobacter lari*. Given the specificity problems observed, the isolated clone 17 can be used as a test for the detection of the *Campylobacter* genus.

Acknowledgments

First, I would like to thank my committee members for their guidance through this project. I was extremely fortunate to have Dr. James Barbaree as a project advisor who made the project possible through funding for my research, and the resolution of all the obstacles I encountered along the way. I am grateful to Dr. Sang-Jin Suh for the constant encouragement, experimental design, discussions of the *Campylobacter* project, as well as his persistent demand for critical thinking. The work within my dissertation would not have been possible without the help and the "Bacterial Pathogenesis" classes of Dr. Stuart Price, who in my opinion is the best lecturer on the campus of Auburn University. I am tremendously grateful to Dr. Vitaly Vodyanoy. It is an extreme honor to have a brilliant scientist like him as member of my research committee. I also want to express my gratitude to my friend and colleague I-Hsuan Chen for her tremendous help in the lab. Lastly, I thank all the undergraduate students for their hard work and perseverance in carrying out the air cabin project, in particular Ashlynne Goodloe, Brian Koonce, Andrew Wenzler, Kyle Owens, Victor Cheng, Joshua Chu and Mason Henderson.

Style manual or journal used: Applied and Environmental Microbiology

Computer software used: Microsoft Word 2010, Microsoft Excel 2010, Microsoft Power Point 2010, IBM SPSS Version 21, SAS 9.2

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Preface

Despite the availability of exceptional molecular diagnostic platforms and pathogen surveillance networks, the health care systems of industrialized nations are constantly overwhelmed by the emergence of new infectious diseases and the re-emergence of diseases that were once contained. The recent globalization trends facilitate the rapid spread of emerging diseases that were once geographically confined, (i.e. severe acute respiratory syndrome (SARS)), creating international epidemics with great health and socio-economic impact.

Airplane travel and trade has contributed significantly to the increase of such disease outbreaks. An exhaustive screening of every passenger and food of animal and plant origin prior to flight is not feasible from an economic stand point. The current efforts of the scientific community on timely detection and preventing the spread of disease by developing vaccines and medicine appear insufficient. Moreover, a great number of re-emerging diseases such as the multi-drug-resistant tuberculosis and methicillin-resistant *Staphylococcus aureus* (MRSA) result from the widespread misuse of antibiotics. A more comprehensive understanding of the etiology, the reservoirs, and the risk of transmission of infectious agents is needed to prevent disease outbreaks during and after airplane flights.

An essential component of a successful intervention strategy relies on the proper identification of a transmission route as a contributor to a disease outbreak. It has been broadly accepted by epidemiologists that surface contamination plays an important role in the transmission of infectious diseases in the food industry and in community settings such as hospitals, schools, and day care. In this context, the cabin of civil aircrafts represents a high risk of infection scenario given the restricted seat space, the high rates of fomite and human contact, and the relatively low air humidity (< 18%). In such a community setting, viral and bacterial pathogens can be deposited on abiotic surfaces or fomites by symptomatic or asymptomatic carriers in body fluids such as saliva, blood, urine, and feces.

Many important human bacterial pathogens are capable of surviving from hours to months on fomites and withstand environmental stress conditions in a viable but non-culturable (VBNC) state. In this state, bacteria maintain low metabolic activity and retain virulence, but grow poorly on routine microbiological media. Currently, sensitive methods able to identify and quantify the VBNC state to assess the level of microbial surface contamination are scarce. In addition, there are no reports on the survival and transmission of bacteria from fomites under typical aircraft cabin conditions. Gathering data on pathogen survivability on abiotic surfaces inside the airplane and studying their transfer efficiency to the hands of passengers would help establish risk assessment models and in turn enable adequate cleaning and disinfection strategies. Therefore, this

dissertation emphasis is on the survival and transmission of two bacterial pathogens, *Escherichia coli* O157:H7 and MRSA, on common airplane cabin surfaces under typical aircraft cabin conditions.

The first chapter reviews existing data on disease outbreaks linked to airplane travel as well as common transmission routes within the aircraft. It also provides a general review of *E. coli* O157:H7 and MRSA. The second chapter highlights the experiments conducted to determine the survival of *E. coli* O157:H7 in the VBNC state when deposited in different simulated human body fluids on surfaces with different physicochemical properties under aircraft cabin conditions. It also presents a contact model based on using pigskin as a surrogate of human hands in an effort to establish transmission rates from surface to skin. The third chapter describes the experiments performed to determine the limits of survivability and transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) on air cabin surfaces employing standard culture methods, as well as the pigskin contact model.

The fourth chapter is dedicated to an unrelated topic, in particular the isolation and characterization of phage peptides specific for *Campylobacter jejuni*. Therefore, the hypotheses of this dissertation are:

I. *E. coli* O157:H7 and MRSA can survive for extended period of time on common surfaces found in the air cabin increasing the risk of transmission to human hands during a commercial air flight.

II. Affinity selected filamentous phage displayed peptides incorporated onto a magnetoelastic sensor can be a specific and rapid alternative of the protocols currently employed to prevent campylobacteriosis and its associated costs.

The objectives of this study are as follows:

1. Assessment of the survival limits of *E. coli* O157:H7 on air cabin surfaces employing:

a) Standard culture methods and qPCR

b) Determination of the efficiency of qPCR-PMA assay to discriminate between viable and dead cells

c) Statistical analysis of the data

2. Assessment of the transmission rates of *E. coli* O157:H7 from air cabin surfaces to hands employing a surrogate pigskin contact model, standard culture methods, and a statistical analysis model

3. Determine the limits of survival and transmission of MRSA on air cabin surfaces by plate counts and statistical analysis

4. Isolation and characterization of phage displayed peptides specific for *Campylobacter jejuni.*

Chapter 1

Literature Review

1. Airplane Travel and Disease

The first scheduled passenger airline service in the United States dates back to the early 1914 (1). The service operated the distance between St. Petersburg and Tampa, Florida with a travel plan of just 21 miles. Today, US and international airlines transport billions of civilians over vast distances around the world in record-breaking times (2, 3). People travel to remote areas of the world where certain pathogens are endemic which causes health problems during and after a commercial air flight (4, 5). The reduction of spatial and time barriers facilitates new infectious diseases to emerge faster and diseases that were once kept under control to re-emerge, increasing the risk of international epidemics (6-8). For instance, the in-flight outbreak of severe acute respiratory syndrome (SARS) in 2002 demonstrated airplane travel can have a significant effect on the passenger's health and can promote global rapid spread of a newly emerging disease (9).

A health assessment of passengers before their flight is not feasible and pathogenic microorganisms can be transferred inside the airplane by the skin, mucous membranes, upper respiratory tract, mouth, and gastrointestinal tract of the passengers.

In the airplane cabin, the risk of infection is high given the limited seat area, the high rates of fomite and human contact, the relatively low air humidity (< 18 %), as well as the presence of immunocompromised individuals and asymptomatic carriers of disease (9-14). Inhaling air with low humidity dries the mucosal membrane and could depress the ciliary movement in the respiratory tract and increase the susceptibility to respiratory viral infections (15). The flight duration is also critical. The length of intercontinental flights is often within the incubation periods of some human pathogens (16).

2. Disease Transmission Routes in the Aircraft

The transmission routes of disease in the aircraft are similar to the patterns of transmission in community scenarios with high human density and close proximity such as households, schools, workplaces, and transport systems (17, 18). In the context of commercial air travel, there are four relevant routes of pathogen transmission: contact, airborne, common vehicle, and vector-borne (19, 20). Contact transmission involves person-to-person contact or indirect when a fomite serves as an intermediary between carrier and susceptible person. Airborne transmission refers to the aerosolization of pathogens from an infected individual and transmission via air to a susceptible host or a fomite in absence of direct contact (21). Common vehicle transmission implies pathogen infection of multiple hosts by food and water through contaminated fomites and lack of hand-hygiene (22). Vector-borne transmission is the spread of disease by arthropod or vermin intermediary (23). These four routes of transmission are not mutually exclusive or specifically defined categories. Infectious agents such as *Escherichia*

coli O157:H7 and MRSA can exploit more than one route to transfer between a carrier and a susceptible host.

3. Common Diseases Transmitted in Airplane Cabins

In the airplane cabin, bacteria and viruses are often dispersed via aerosolization in wet droplet nuclei of mucous secretions and propelled when an infected host sneezes or coughs. (14, 24). However, the formation of droplet nuclei is not a prerequisite for this type of transmission. The risk of airborne disease in the aircraft cabin is largely based on reports of outbreaks due to SARS and *Mycobacterium tuberculosis* (9, 14). Three factors appear to be important: 1) the restricted seat space, 2) the length of the flight (> 8 h), and 3) an effectively working ventilation system (11, 14, 25). Airborne transmission risk increases if the recirculation of air in laminar flow is disrupted. The laminar pattern divides the air flow into sections and reduces the front-to-back airflow limiting the spread of airborne particles in the cabin. The importance of this type of ventilation was exemplified by an influenza outbreak when passengers were kept aboard with an inoperative ventilation system (26). Furthermore, the air is filtered by high efficiency particulate air filters (HEPA), which eliminate infectious particles. The risk of transmission increases if the HEPA filters are not working (25).

3.1. Tuberculosis (TB)

Tuberculosis is a chronic disease caused by *M. tuberculosis*. Most infections are asymptomatic and latent in nature. One in ten latent infections progresses to active disease and, if untreated, kills more than 50% of the infected

(27). The onboard transmission of *M. tuberculosis*, has been suspected in multiple reports; however, it was confirmed only in two studies (14, 28). In the first case in 1992, an infected flight attendant transmitted the disease to crew members and passengers (28). The second event was caused by a passenger with pulmonary tuberculosis traveling from Baltimore to Chicago and then on to Honolulu. Four out of fifteen passengers seated within two rows of the infectious passenger tested positive with the tuberculin skin test (14). The close contact with a contagious person and the length of the flights were the two common risk factors in both outbreaks. Of particular concern is the potential transmission to passengers of Multi-drug resistant Tuberculosis (MDR-TB), which is challenging and costly to treat due to its resistance to most antibiotics (14). Risk assessment studies have estimated the overall probability of infection to be one in one thousand if a symptomatic carrier is present, a probability comparable to the risk of transmission in confined spaces (29, 30).

3.2. Meningococcal Disease

Meningococcal disease is a life-threatening infection caused by the bacterium *Neisseria meningitidis.* The disease occurs usually after direct contact with respiratory secretions, and is associated with high morbidity and mortality rates (31). Air-travel associated meningococcal infection is defined by two criteria: symptoms appearing fourteen days after the date of air-travel and flight duration of at least eight hours. From 1999 to 2001 the US Centers for Disease Control (CDC) received 21 reports of suspected onboard meningococcal disease.

In all cases a contagious person was present during the flight, but no secondary cases of the disease followed (32).

3.3. SARS

SARS is an unusual pneumonia caused by a coronavirus and is associated with significant morbidity and mortality (7). Evidence suggests that transmission occurs predominantly by contact with respiratory droplets from an infected person or by direct contact with contaminated hands or fomites. The most notable SARS transmission onboard occurred in a flight from Hong Kong to Beijing in March 2003. Twenty-two people were infected during the flight with a sequel of 300 secondary cases. The outbreak was linked to a suspected malfunctioning cabin filtration system rather than the flight duration (9).

3.4. Influenza

Transmission of influenza onboard has been reported in several studies (33-35). The close proximity between seats and an inoperative ventilation system were the most probable causes. However, the onboard transmission is of lesser concern compared to the potential of the aircraft to serve as a vector for rapid global spread of influenza strains worldwide (34).

3.5. Measles

Measles is a serious viral disease transmitted by direct contact with infectious droplets or by airborne transmission. Due to the mass vaccination of the population in industrialized countries, measles is endemic only in certain parts of

the world and measles infection in air travelers may be considered a low-risk event (36, 37).

4. Common Vehicle Transmission

Common vehicle transmission onboard involves the spread of pathogens by the fecal-oral route via contaminated food. In the modern era, foodborne illness associated with air travel is rare due to the introduction of hazard analysis and food-safety standards in the flight catering industry (16). Outbreaks during long intercontinental flights (13–14 h) are of particular concern given that certain pathogens such as *S. aureus* have an incubation period of less than the duration of the flight (16). Historically, different serotypes of *Salmonella enterica* account for the majority of food-borne infections, 39.5%, followed by *S. aureus* with 16.3% of all reported outbreaks as well as sporadic cases caused by *Vibrio* spp. and *Shigella* spp. (16, 38). The largest food-borne outbreak described was due to consumption of orange juice contaminated with a Norwalk-like virus, isolated from the fecal samples of thirty passengers (39).

5. Vector-Borne Transmission

The most frequently reported vector-borne transmitted illnesses related to air travel are malaria, dengue fever, and yellow fever. These tropical diseases can be transmitted by mosquitoes and are often imported with exotic animals (23). The vaccination requirements prior traveling to tropical countries, as well as disinfection of the aircrafts at the airports, have prevented the occurrence of outbreaks (40).

6. Direct and Indirect Contact Transmission

Direct transmission onboard is based on the assumption of relatively proportional mixing between susceptible and infected individuals. Direct contact transmission is difficult to define because the event of infectious contact is unclear when compared to the other transmission routes. This paradigm is often experienced when modeling transmission routes of *M. tuberculosis* and SARS in environments such as the airplane cabin (39). Large droplets from sneezes or coughs can be spread over short distances but they can also settle and be transmitted indirectly by a fomite to a susceptible host. The role of fomites commonly found in the aircraft cabin has only been hypothesized as a possible route of transmission (41). Even though specific data regarding the implication of fomites onboard under typical aircraft cabin conditions are absent, the role of surface contamination and the consequent transmission of bacteria, bacterial spores, and viruses to susceptible hosts is supported by substantial evidence, both in nosocomial and community settings (42-44).

7. Definition of Fomites

Abiotic surfaces or fomites consist of porous and nonporous materials with variety of physicochemical properties that can act as reservoirs and intermediaries for indirect contact transmission of infectious diseases. Fomites become contaminated by direct contact with hands, body fluids, or contact with aerosols shed by symptomatic and asymptomatic carriers of pathogens. As a consequence, fomites with settled viruses, bacteria, or fungi are the origin of the transmission to humans or uncontaminated fomites. At present, numerous studies

report of the development of surfaces which release antimicrobial compounds or metal ions such as silver and copper which kill bacteria upon contact. Developing antimicrobial surfaces is of great importance in reducing the incidence of nosocomial infections (45).

8. History of Fomites and their Role in Disease Transmission

The role of fomites in indirect disease transmission was first recognized in 1546 by the Italian physicist and scholar Girolamo Fracastoro (46). Besides identifying direct physical contact as a cause for syphilis and gonorrhea, and airborne transmission as a cause for tuberculosis and smallpox, he also observed that some diseases were transmitted by fomites such as clothing (from the Latin fomes, meaning "tinder"), that had been in contact with the sick (46). Contaminated fomites (blankets) were used as biological warfare by the British colonialists to fight the indigenous population of the New World. As a consequence, smallpox epidemics devastated Native Americans for almost a decade (47). The awareness of fomite connection to disease transmission prevented outbreaks due to merchandise importation. In 1835, the British Privy Council quarantined all ships carrying cotton from Egypt during a plague outbreak in Alexandria, avoiding an outbreak of similar etiology that had occurred in London in 1665 (48). In the modern era of Microbiology, Owen Hendley and Jack Gwaltney in 1973 provided (for the first time) substantial evidence that contaminated fomites can serve as a secondary source of disease transmission, and also described the steps required for the occurrence of this phenomenon. Their study showed that rhinovirus was shed from an infected host in the

environment. The virus was capable of surviving on an environmental surface and the contaminated surface transmitted the pathogen through contact with a susceptible host (49). These findings drove the scientific interest in elucidating the relevance of fomites for the high incidence of certain nosocomial pathogens such as the respiratory syncytial virus (RSV). Hall and Douglas Jr confirmed the findings of Hendley and Gwaltney of indirect contact for the RSV transmission. They also demonstrated that RSV survives on surfaces and the indirect transmission between surfaces and hands of susceptible individuals occurs when they touch their nose or eyes (50, 51). In a later study Hall showed large droplet contact and indirect contact with fomites were more efficient routes for RSV transmission than aerosolization (52).

9. Factors Affecting the Persistence of Pathogens on Fomites

Ever since the discovery of Hendley and Gwaltney, numerous studies have been conducted to monitor the survival time of pathogens on surfaces in hospital and community settings, as well as in the food manufacturing industry. The main purpose is to provide data that would aid in applying adequate surface disinfection and determine the health risk. A review article of Kramer (2006) has compiled experimental data of the most important nosocomial pathogens (viruses, bacteria, and fungi) and their survival on hospital surfaces (42). Factors such as desiccation and relative humidity, temperature, ultraviolet light exposure, concentration of the initial inoculum, and surface substrate have different degrees of impact depending on the pathogen and the experimental methods employed to study those stressors (42).

The majority of nosocomial and foodborne bacterial pathogens are considered mesophilic in nature and therefore they tolerate well temperatures between 20 and 45 °C. Several studies have shown that low temperatures (4°C - 6°C) enhance the persistence of important human pathogens such as methicillin-resistant *S. aureus* MRSA and *E. coli* O157:H7 (53, 54). However, temperature is not the most deleterious factor affecting their survival in indoor environments. Bacterial pathogens must withstand dehydration through air drying, without being killed, in order to be transmitted by fomites. The air humidity in the airplane cabin (< 18%) and other indoor environments such as hospitals is relatively low (40-65%). Therefore bacteria must adapt and develop desiccation tolerance (12, 55). Desiccation tolerance is the ability of an organism to reach desiccation up to a state of equilibrium when exposed to moderate or extremely dry conditions, and must be able to recover after rehydration (56).

The most desiccation tolerant non-spore forming bacteria are Gram-positive bacteria such as *S. aureus* (57). It has been hypothesized that Gram-positive bacteria are more resistant to desiccation given the thicker cell wall rich in peptidoglycan (58). A common adaptation strategy of bacteria to desiccation is the uptake and accumulation of non-reducing disaccharides such as sucrose and trehalose (59). These sugars act as osmo-protectants by replacing the water and are involved in the formation of a vitreous cytoplasmic matrix (60). Another mechanism used by bacteria to cope with desiccation is the rapid synthesis of heat shock proteins (HSP). HSP stabilize denatured proteins and prevent their aggregation in an adenosine-triphosphate (ATP) independent manner (61). Gram

positive species such as *Deinococcus radiodurans* uptake and accumulate Mn^{2+} and Fe^{2+} that act as antioxidants against reactive oxygen species (ROS) during desiccation (62, 63).

The dehydration increases the ionic concentration which leads to the formation of ROS. ROS are generated during respiration and accumulate due to malfunctioning or inactive peroxide scavenging enzyme (64). The presence of ROS can damage proteins by causing modifications of amino acid side chains, formation of cross-links between proteins, and fragmentation of the polypeptide backbone. In addition, ROS can modify bases and sugars in deoxyribonucleic acid (DNA), leading to DNA chain breaks, and cause lipid peroxidation in cell membranes. (65). Bacteria have developed mechanisms to repair damaged DNA. For instance, the RNA Polymerase sigma factor S (RpoS) is involved in the regulation of acid, heat, and salt tolerance (66). It also modulates the expression of the enzyme recombinase A (RecA) involved in DNA repair as well as the expression of the DNA binding protein (Dps) (67, 68). It has been experimentally determined that RecA, Dps, and RpoS play an important role in *E. coli* O157:H7 survival during desiccation (69). In addition, to eliminate ROS, *E. coli* employs superoxide dismutases and catalases, the expression of which is modulated by the oxidative stress regulons OxyR and SoxRS, as well as by RpoS (70). Another mechanism employed by bacteria to overcome such extreme environmental stressors, is a dormancy state known as a "viable but nonculturable (VBNC) state".

10. Viable But Non-Culturable (VBNC) Bacteria

Traditionally bacterial viability is defined by a bacterium's ability to form colonies on solid agar plates under optimal conditions. In contrast, when in the VBNC state, non-spore forming bacteria are unable to grow on standard culture media. It is considered a transient long-term survival and protection mechanism (71, 72). Bacteria become VBNC in response to harsh environmental conditions such as starvation, abnormal growth temperature, oxygen, pH, food preservatives, heavy metals, and UV light (71-73). The cells in this state are morphologically smaller, undergo cell wall modifications, exhibit low metabolic activity, and exhibit a particular gene expression profile, but may remain virulent and can recover viability under favorable conditions (74-76). For example, the VBNC of *Vibrio harveyi* is not lethal when inoculated into *zebra* fish because the hemolysin gene is not expressed. However, after resuscitation, the bacteria were lethal indicating *V. harveyi* retained its virulence (77). The capacity of certain human pathogens to enter VBNC is an important health concern. For instance, the latent phase of *M. tuberculosis* and the recurrence of the tuberculosis infection years after are now believed to be consequence of VBNC bacteria residing inside solid granulomas (73, 78). In addition, it has been demonstrated that the VBNC state of nosocomial infections caused by *Enterococcus faecalis* renders the pathogen resistant to vancomycin concentrations up to 500 times the minimum inhibitory concentration (MIC) (79).

11. Methods for Detection of VBNC bacteria

11.1. Traditional Methods

The conventional enumeration method based on plating and counting colony forming units (CFU) is labor-intensive, time-consuming, lacks sensitivity for pathogens with low infectious dose, and is inadequate for the detection of the VBNC state (71, 80). In addition, the presence of CFU on a plate does not reveal the viability of individual cells; it is rather a rough estimation of a fraction of cells capable to grow and divide. Other culture based methods with similar limitations are the membrane filter and most probable number (MPN) (81). The ability of bacteria to adapt and withstand environmental stressors in the VBNC state requires more accurate and sensitive detection methods allowing the assessment of environmental samples such as air, water, soil, and food for the possible presence of viable pathogens.

11.2. Membrane Integrity Methods

The techniques based on membrane integrity employ fluorescent microscopy and fluorescent dyes such as SYTO9 and Propidium Iodide. These stains have high affinity for the DNA. Propidium Iodide penetrates only cells with damaged membranes, while SYTO9 penetrates all bacterial membranes. As a result, viable cells stain green, while the dead cells and cells which have lost membrane integrity appear red (82). A limitation of the technique is that it relies exclusively on the membrane integrity of the VBNC cells. However, the permeability of the bacterial cell might not be an indication whether the cell is viable or dead. Viability can also be defined by the ability of the bacterium to

metabolize compounds, maintain a proton gradient, or maintain replication capability. Depending on the degree of injury, certain Gram-positive and Gram-negative species sometimes stain in intermediate colors, depending on the extent of stress, and it is difficult to get clear results of their viability status (82).

11.3. Flow Cytometry

Flow cytometry (FCM) is a technique employing analytical instruments operating with laser beams which allow the biophysical status assessment of individual cells. The method is usually based on fluorescent staining or fluorescently labeled antibodies. FCM is sensitive enough to detect changes in outer membrane proteins, lipopolysaccharides, and surface antigens that vary depending on the environmental conditions or the physiological state of the organism; thus, providing information with regard to viability and virulence (83, 84). Major disadvantages of this technique are the high cost of the flow cytometric instrumentation and the need for well-trained users to optimally use the instrument.

11.4. Fluorometric Methods

Fluorometry measures the emission of fluorescence by charged compounds exposed to electromagnetic radiation (85). Fluorometric methods allow the evaluation of the viability of bacteria based on their metabolic activity, membrane potential, membrane integrity, or intracellular pH in real-time under user-defined conditions (e.g. nutrients, temperature, pH) while employing fluorescent substrates. Bacteria are labeled in a suspension and exposed to excitation light with an appropriate wavelength. For example, ethidium bromide

(EB) emits weak fluorescence outside cells, but is highly fluorescent in hydrophobic environments such as the periplasmic space of Gram-negative bacteria. The fluorescent intensity of EB has been used for real-time monitoring of the accumulation and efflux of substrates in bacterial cell suspensions (86). Another example is the Bis-trimethine oxonol-DiBAC$_4$ (1,3-dibutylbarbituric acid) (Molecular Probes, Inc.), an anionic stain that allows the analysis of acid-treated bacteria and reveals changes in the membrane potential, depolarization or hyperpolarization (87). These methods provide accurate data but require time, expensive equipment for the fluorescent measurement, and are labor intensive.

11.5. Nucleic Acid Methods

These methods are used to detect sequences of nucleic acids, either DNA or RNA, by real-time quantitative PCR (qPCR) and real-time reverse-transcription PCR (qRT-PCR). Both methods detect, amplify, and simultaneously quantify specific nucleic acid sequences based on a/their/the fluorescent signal. (88, 89). The qRT-PCR is based on the assumption that messenger RNA (mRNA) is only present in metabolically active cells and is not found in dead cells, given mRNA has a half-life of less of than 1 minute (90). DNA detection methods employ fluorescent dyes that intercalate double-stranded DNA (i.e. SYBR Green) or DNA probes carrying a quencher and a fluorescent reporter (i.e., 6-carboxyfluorescein – FAM; TaqMan aproach) (91). The quantity is measured either as an absolute number of copies or a relative number when normalized with a standard curve of a reference gene. Unlike conventional PCR, the use of real-

time fluorescent measurement gets around the necessary electrophoretic analysis to demonstrate the presence of amplified product. Nonetheless, the detection and quantification of VBNC bacteria solely by qPCR is of little value because the method is unable to discriminate DNA from live and dead bacteria (92). DNA is a relatively stable molecule and it could persist in a given sample long after all viable cells have been killed by environmental stress. Recently, the real-time quantitative PCR technique has been optimized by combining it with nucleic acid intercalating dyes such as ethidium monoazide (EMA), and propidium monoazide (PMA) that inhibit amplification of DNA from dead cells (93). Both molecules contain an azide group and selectively penetrate damaged membranes of dead cells and covalently link to DNA by photolysis under intense visible light, preventing the amplification of targeted sequences from dead cells (93). Another chemical, released by Biotecon Diagnostics under the name reagent D has been reported to act in an analogous mode to EMA and PMA. However, it has been demonstrated that EMA and reagent D are not optimal in use of VBNC quantification (93, 94). Although both EMA and PMA intercalate the DNA of dead cells, PMA penetrates fewer viable cells and VBNC with undamaged membranes primarily due to its two positive charges (93). The real-time qPCR method combined with PMA (qPCR-PMA) has been successfully optimized and employed to detect VBNC cells of important foodborne bacterial pathogens such as *Salmonella*, *Listeria monocytogenes*, and *Campylobacter jejuni*, as well as *E. coli* O157:H7 in different types of food (95-97).

12. *Escherichia coli* O157:H7 Overview

Escherichia coli are Gram-negative, oxidase-negative, non-spore forming rods that are members of the Enterobacteriaceae famly and can either be non-motile or motile by peritrichous flagella. They are bile tolerant, non-fastidious organisms, routinely grown on standard media at 37 °C. These bacteria are facultative anaerobes and can use citrate, nitrate and fumarate as alternative electron acceptors. Also, under anaerobic conditions, *E. coli* can carry out mixed-acid fermentation, producing lactate, succinate, acetate, carbon dioxide and hydrogen gas (98).

Most *E. coli* strains are harmless and beneficial members of the intestinal tract of humans and animals. However, certain strains of the species have acquired virulence genes that enable them to cause disease in humans and animals. These *E. coli* strains are divided into intestinal pathogens causing diarrhea and extraintestinal pathogens that can cause urinary tract infections (UTI), meningitis and septicemia (99). The diarrheagenic *E. coli* strains are divided in pathotypes based on different biochemical and pathogenicity characteristics. These are enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC) or Vero toxin-producing / Shiga toxin-producing *E. coli*, enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (100). Each of these pathotypes exhibit specific colonization and virulence factors that lead to colonization of the intestinal mucosa and diarrhea with different clinical symptoms and outcomes of disease (101). The Shiga toxin-producing *E. coli* (STEC) refer to those strains that

produce at least one of the Shiga toxins (Stx1) or (Stx2) and have the same clinical, epidemiological and pathogenic features associated with the prototype strain *Escherichia* O157:H7 responsible for most of the outbreaks in the United States (102).

12.1. Clinical Manifestation and Incidence

The main clinical manifestation of the infection is severe diarrhea that may last 2-8 days, commonly known as hemorrhagic colitis (HC). In 10 to 15% of the patients with HC, the infection progresses to Hemolytic Uremic Syndrome (HUS). HUS is characterized by progressive renal failure associated with microangiopathic hemolytic anemia and thrombocytopenia (103). Commonly, children less than 5 years, old and immunocopromised individuals are the most susceptible to these severe complications. Approximately 40% of the patients with HUS develop long-term renal dysfunction, and about 3 to 5% of patients die during the acute phase of the disease (104). Infections caused by certain non-O157:H7 STEC serotypes can also cause HC and HUS (105). An estimated 63,153 cases of O157 and 112,752 of non-O157 STEC illnesses are recorded in the US annually (102). The clinically significant group known as the “big six” (O26, O45, O103, O111, O121, and O145) as identified by the Centers for Disease Control and Prevention (CDC) is the cause of over 70% of non-O157 STEC-associated illness (106).

12.2. Genome characteristics

Sequencing studies of O157:H7 and non-O157 strains (O26, O103, and O111) revealed the pathogenic variants share a 4. 1 Mb backbone sequence conserved in all *E. coli* strains, but have much larger genomes due to newly acquired virulence and fitness genes (5.5–5.9 Mb) (107-110). The *E. coli* O157:H7 lineage has gained new sequences such as the virulence plasmid pO157 (111), Shiga toxin converting phages (112), pathogenicity islands containing sequences of the enterocyte effacement (LEE) and the arginine translocation system (113, 114), as well as adhesion factors for attachment and biofilm formation (115). A comparison with the nonpathogenic *E. coli* K12 has also shown genomic reduction of 0.53 Mb of DNA in *E. coli* O157:H7, suggesting the loss of certain sequences has also contributed for the evolution of the pathogen (107).

12.3. Virulence Factors

12.3.1. Shiga Toxins (Stxs)

The Shiga toxins are the primary virulence factors of STEC strains and are the main cause of HUS in humans (116). These toxins belong to the AB_5 family of compound toxins with pentameric ring-shape composed of five identical B subunits (B_5) non-covalently attached to the A subunit (A_1). The B_5 subunit interacts with globotriaosylceramides (Gb3s) on the surface of human glomerular endothelial cells and other cells containing the receptor, promoting the internalization of the A_1 subunit (117). The A_1 subunit has RNA N-glycosidase

activity which shuts down protein synthesis by cleaving a specific adenine base from the sarcin/ricin loop of the 28S rRNA, triggering cell death (118, 119).

12.3.2. The Locus of Enterocyte Effacement (LEE)

In order to attach and colonize the host intestinal cells, STEC require the formation of attaching and effacing (A/E) lesions enabled by a type III secretion system (T3SS) that subsequently injects a cocktail of effector proteins. The sequence of the T3SS is encoded on a large (42kb) pathogenicity island located on the chromosome called the locus of enterocyte effacement (LEE) (120). The translocation of effector proteins initiates the degradation of the host cell microvilli and the reorganization of host cell cytoskeleton by the accumulation of polymerized actin directly beneath the adherent bacteria (pedestal formation) (120). The formation of the A/E lesion triggers the translocation of other effector proteins LEE and non-LEE-encoded which interfere with host cell signaling, alter transcription and modulate the immune response (121-123).

12.3.3. The pO157 Plasmid

Another distinct feature of *E. coli* O157:H7 is the presence of a plasmid called pO157 with a size range of 92 to 104 kb. Sequencing studies have shown that this non-conjugative F-like plasmid integrates fragments from evolutionally different species such as transposons, prophages, insertion sequences (IS), and parts of other plasmids (124, 125). The role of pO157 has not been elucidated, however this plasmid contains genes that might be involved in the pathogenicity *E. coli* O157:H7 such as a hemolysin (*ehxA*) (126), a periplasmic catalase

peroxidase (*katP*) (127), a type II secretion system apparatus (*etp*) (128), a serine protease (*espP*) (129), a putative adhesin (*toxB*)(130), a zinc metalloprotease (s*tcE*) (131) and an *eae* conserved fragment (*ecf*) (132).

12.3.4. Identification and Detection Methods for *E. coli* O157:H7

Laboratory confirmation of STEC infection can be achieved using culture media, immunoassays, cell toxicity assays and PCR (87–88). Screening of O157 relies on the strain's inability to utilize sorbitol rapidly, leading to the use of sorbitol-MacConkey agar (SMAC) as a differential medium. More specific media have also been developed such as Rainbow agar, CHROMagar®, and O157: H ID agar that are able to recover O157 along with sorbitol-fermenting O157 and non-O157 strains (70, 88). PCR offers the fastest and most reliable method for detection of STEC which, similar to immunoassay tests, can be used directly with stool samples as well as isolated colonies. Depending on the primers used, PCR can distinguish between *stx*1 and *stx*2 and detect *eae* and enterohemolysin (*hly*) genes (88).

12.3.5. Reservoirs and Transmission

The main route for *E. coli* O157:H7 transmission is the fecal-oral route. Contaminated food and water are responsible for the majority of the food outbreak-related illnesses in the United States (99). Cattle and other ruminants are the major reservoirs and contact with their feces is an important source of human illness (106). Meat products can become contaminated during slaughter of colonized animals, whereas vegetables become contaminated by manure used as

fertilizer or through contaminated irrigation water (107). Human illness has been caused by food such as uncooked ground beef, sausage, dairy products, apple cider, lettuce, spinach, and sprouts (108). Contact with farm and zoo animals or their feces also represents an important transmission risk (109). Person-to-person transmission is not uncommon and has been implicated in up to 19% of the STEC O157:H7 outbreak cases (110). Asymptomatic shedders have been reported as the primary source of person-to-person transmission outbreaks in day care facilities (111). In addition, several reports have shown that *E. coli* O157:H7 can survive on abiotic surfaces with different physicochemical properties under a variety of environmental stressors for extended intervals of time which in turn can facilitate its transmission via aerosols or direct contact (112-118). Given the importance of airplane travel and the contribution of contaminated fomites in previously reported outbreaks, we believe it is critical to investigate the survival and transmission of *E. coli* O157:H7 on surfaces under typical aircraft cabin conditions.

13. Methicillin-resistant *Staphylococcus aureus* (MRSA) Overview

Methicillin-resistant *Staphylococcus aureus* (MRSA) are clinical isolates of *S. aureus* that are resistant to β-lactam and other non β-lactam antibiotics (133). This pathogen belongs to the family of *Staphylococcaceae*. The genus *Staphylococcus* includes 42 species and subspecies (134, 135). *S. aureus* is a Gram-positive, coccus-shaped non-spore forming, non-motile, catalase-positive and coagulase-positive bacterium growing in grape-like clusters. It is a facultative anaerobe that can respire in the presence of oxygen and has the ability to grow

under low-oxygen conditions by fermentation or nitrate respiration (136). The organism can tolerate high salt concentrations (10-15%) and temperatures of up to 45°C, but optimal growth occurs at temperatures of 25°C to 45°C and at pH levels of 4.8 to 9.4 (136, 137). Besides *S. aureus*, which is coagulase positive, there are four other coagulase-negative species of clinical importance to humans: *S. epidermidis*, *S. haemolyticus* and *S. lugdunensis* (138).

Before the launch of penicillin as a treatment of *S. aureus* infections, the mortality rate in humans was over 80% (139). However, in 1942, only two years after the introduction of penicillin for medical use, the first penicillin-resistant *S. aureus* isolates were observed (140). The effectiveness of semi-synthetic beta-lactams such as methicillin developed in 1959 was short-lived and less than two years after their introduction methicillin-resistant *S. aureus* emerged (141). Since 1960, MRSA became a global epidemic and 80% of all clinical *S. aureus* isolates have been methicillin-resistant. Just like its predecessor, MRSA is a daunting pathogen equipped with virulence factors capable of causing life-threatening disease with few options for medical treatment.

13.1. Genome Characteristics

The genome of *S. aureus* consists of a single circular chromosome of approximately 2.9 megabase pairs (Mbp) in size with a relatively low G+C content (142). High-throughput sequencing studies have shown that approximately 75% of *S. aureus* genome is highly conserved between strains and contains all the housekeeping genes essential for bacterial growth and virulence

(143, 144). The rest of the genome is composed by genetic elements such as pathogenicity islands, bacteriophages, chromosomal cassettes, genomic islands, plasmids, transposons and prophages transferred horizontally between strains and believed to enhance antibiotic resistance, virulence and cause particular clinical syndromes (142-144).

13.2. Mechanisms of Antibiotic Resistance

Two are the main mechanisms responsible for the broad-spectrum beta-lactam resistance in MRSA. The first is due to the presence of the *mecA* gene. This gene encodes for penicillin-binding protein 2a (PBP2a). This protein has a low affinity for beta-lactams and provides transpeptidase activity for cell wall synthesis at beta-lactam concentrations that inhibit the beta-lactam-sensitive PBPs normally produced by *S. aureus* (144). The second mechanism common for most MRSA is the production of β-lactamase that inactivates β-lactams (145). The *mecA* gene is located on a mobile genetic element (MGE) called the staphylococcal cassette chromosome *mec* (SCC*mec*) (146, 147). The SCC*mec* encodes the *mec* gene complex and the *ccr* gene complex. The *mec* gene complex consists of *mecA*, regulatory genes and associated insertion sequences. According to these components, MRSA has been classified into six different classes, i.e. I, II, III, IV, V and VI. The *ccr* gene complex encodes recombinases (*ccrC* or the pair of *ccrA* and *ccrB*) mediating integration and excision of SCC*mec* into and from the chromosome (146, 147). In the past it has been hypothesized that all MSRA clones have a common methicillin-susceptible *S. aureus* (MSSA) ancestor which acquired SCC*mec* only once (148). Currently, a more commonly accepted theory

is the multi-clone theory which suggests that the SCC*mec* element was introduced into different *S. aureus* lineages multiple times (149).

In addition to the resistance to β-lactams, MRSA can be resistant to other antimicrobials such as aminoglycosides, tetracyclines and heavy metals due to resistance genes integrated into the SCC*mec* as plasmids or transposons (150). MRSA isolates have also developed resistance to fluoroquinolones due to a mutation in the DNA gyrase genes, efflux pumps and reduced permeability (151).

In 1997, a vancomycin intermediate-resistant *S. aureus* (VISA) was reported from Japan (152). Mutations and altered expression of certain genes, resulting in a thickened cell wall that prevents vancomycin from properly functioning are believed to have promoted the resistance (153). Vancomycin-resistant MRSA (VRSA) was first noticed in 2002 in Michigan (154). In addition to the *mecA* gene, VRSA strains contain a plasmid-borne transposon Tn*1546* with a *vanA* gene cluster acquired through conjugation from a glycopeptide-resistant *Enterococcus* strain (155, 156).

13.3. Identification and Detection Methods MRSA

The identification of MRSA can be achieved through standard microbiological methods, immune assays and molecular methods such as PCR. The standard microbiological methods test for the phenotypic characteristics of the organism. Commonly performed methods are the Gram-staining, catalase, coagulase, culture on mannitol salt agar, blood agar BBL CHROMagar MRSA (at 37º C for 18 to 24h), as well as fermentation (oxidase) and tube test (coagulase).

Clinical isolates are tested for antibiotic resistance using the Kirby Bauer disk diffusion test (145-147). These identification tests can be achieved faster by automated systems such as the MicroScan also WalkAway (©Siemens AG) and Vitek 2 **(**bioMérieux, Inc.) (148). Another relatively rapid and inexpensive method is the penicillin binding protein 2a latex agglutination (PBP-LA) assay (Denka Seiken Co., Japan/Oxoid Ltd., United Kingdom). This test has been approved by the Food and Drug Administration (FDA) a for the identification of methicillin resistance in *S. aureus* from culture (149). However, these tests suffer from lack of sensitivity and specificity and commercial PCR methods are under development and optimization to circumvent those hurdles. The IDI-MRSA assay (GenOhm, San Diego, CA) is a multiplex qualitative real-time PCR assay for detection of MRSA from nasal swabs. It employs five primers targeting SCC*mec* right-junction sequences corresponding to SCC*mec* types I, II, III, IVa, IVb, and IVc allowing the differentiation of *mecA*-positive *S. aureus* from coagulase-negative staphylococci (150). Another commercially available real-time PCR method is the GeneXpert MRSA/SA BC Assay (Cepheid, Sunnyvale, CA) identifies MRSA based on detection of the *spa* and *mecA* genes and the junction between the staphylococcal cassette chromosome that harbors *mecA* (SCC*mec*) and the *S. aureus* genome (*orfX*) (151).

13.4. Hospital acquired MRSA

Hospital acquired MRSA (HA-MRSA) is among the leading nosocomial pathogens in the United States (152). It is a frequent cause of skin and soft tissue infections (SSTIs), endovascular infections, pneumonia, septic arthritis,

endocarditis, osteomyelitis, foreign-body infections, and sepsis (152). Infections caused by HA-MRSA strains are commonly linked with history of healthcare exposure within the past year. These strains exhibit the following characteristics: 1) cause invasive infections, 2) resistance to clindamycin and fluoroquinolones, 3) they are SCC*mec* type I, II, or III, and 4) lack the Panton-Valentine leukocidin (*pvl)* gene (133).

Historically, MRSA infections were considered limited to the hospital environment, and patients treated at these facilities were among the population at risk. As a consequence, the study of MRSA outbreaks has concentrated primarily on health care facilities and persons such as elderly in nursing homes, the immunocompromised, and drug users who are at an obvious risk (153-155). However, in the past two decades the incidence of MRSA have increased drastically as a result of the emergence of community-associated MRSA (CA-MRSA) strains, and the spread of disease to healthy individuals with no prior history of hospitalization (133). Skin and soft-tissue infections are the most common type of CA-MRSA infections (156).

13.5. Community associated MRSA

Community associated MRSA **(**CA-MRSA) infections are not related to hospitalization in the past year but are linked to community settings where healthy individuals are in close contact. These strains display the following characteristics 1) cause soft tissue infections (SSTI) or pneumonia, 2) are variably resistant to clindamycin and fluoroquinolones, 3) they are SCC*mec* type IV, V, or VI, and 4) contain the *pvl* gene (133). In the past decade the distinction between HA- and

CA-MRSA has become increasingly difficult given the reports of CA-MRSA infections in healthcare facilities in the US and other countries (57-60).

13.6. Pathogenesis and Virulence

13.6.1. Adhesion

The first step of infection is initiated by the adhesion of the pathogen to the extracellular matrix of the host. During this phase *S. aureus* has to evade innate immune response of the host defenses. The pathogen possesses a polysaccharide capsule and a thick peptidoglycan hypothesized to block phagocytosis by masking the complement factor C3b bound to the cell wall (157). The zwitterionic capsule can also cause the formation of abscesses (158). Phagocytosis is also impeded by a surface protein termed staphylococcal protein A (Spa) encoded by the *spa* gene, present in most *S. aureus* strains (159, 160). This surface protein is a 42 kDa protein composed by five approximately identical Ig-binding domains, a polymorphic region X and C-terminal cell wall attachment sequence (161, 162). Protein A binds to the heavy chain within the Fc region of most immunoglobulins and also within the Fab region in the case of the human VH3 family. This interaction disrupts opsonization because immunoglobulins bind to the bacterial surface in the wrong orientation (163).

The adhesion to the host tissues is achieved by other surface proteins termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), including fibronectin-binding protein, fibrinogen-binding protein, collagen-binding protein, other adhesins, and anti-opsonins (137). MSCRAMMs mediate adherence to host tissues, bacterial cells, abiotic surfaces

and play a key role in the initiation of endovascular, bone, joint and indwelling-device infections (164, 165).

13.6.2. Extracellular Enzymes

The invasion of host tissues is supported by a variety of extracellular enzymes. For instance, coagulase reacts with blood prothrombin to form a staphylothrombin complex. The staphylothrombin converts fibrinogen to fibrin which aids the bacterium to cover itself with fibrin reducing the susceptibility to host defenses (166, 167). On the other hand, the enzyme staphylokinase degrades the fibrin reacting with plasminogen to form plasmin which is a serine protease digesting the fibrin clots, allowing the spread of the pathogen deep in the host tissue (168). The spread of *S. aureus* in the host tissues is also promoted by the enzyme hyaluronidase and collagenases that degrade the extracellular matrix (167, 169, 170).

13.6.3. Toxins

S. aureus also holds a large armamentarium of toxins which induce host tissue damage. Toxin mediated diseases include staphylococcal scalded skin syndrome (SSSS), toxic shock syndrome (TSS), necrotizing pneumonia and staphylococcal food poisoning.

13.6.4. Staphylococcal Scalded Skin Syndrome (SSSS)

Staphylococcal scalded skin syndrome is a disease characterized by superficial blisters and desquamation of the skin epidermis. The disease is observed in newborns and young children, but may also affect immune

compromised patients and adults with renal failure. (181). The cause of SSSS are the exfoliative toxins (ETs) produced by *S. aureus*. Three isoforms of exfoliative toxins A (ETA), B (ETB) and D (ETD) have been identified (182, 183). These ETs are glutamic acid-specific serine proteases that cleave desmogleins, proteins that are structural part of the desmosomes keeping the epidermal keratinocytes collective structure by anchoring the intermediate filaments of one cell to another (184, 185). The loss of keratinocyte adhesion causes the development of IgG autoantibodies targeting the desmogleins causing the subsequent epidermal lesions (184, 185).

13.6.5. Toxic shock syndrome (TSS)

TSS is caused by toxic shock syndrome toxin 1, which acts as a potent superantigen (186, 187). Menstrual TSS is typically associated with use of tampons among previously healthy women (188). Non-menstrual TSS may result from any primary staphylococcal infection, or from colonization with a toxin-producing strain of *S. aureus* (189). The symptoms of TSS include high fever, hypotension, rash and the involvement of multiple organ systems (189, 190).

13.6.6. Necrotizing pneumonia

The Panton-Valentine leukocidin (PVL) toxin, characteristic for CA-MRSA, causes necrotizing pneumonia and severe skin disease. The PVL toxin is encoded on a prophage integrated in the genome of the pathogen. PVL kills host leukocytes by forming pores in their membranes through which cell contents leak

(133, 140). This toxin also triggers neutrophils to generate reactive oxygen species, which in turn enhances the host tissue injuries (140, 171).

13.6.7. Staphylococcal food poisoning (SFP)

The disease results from the consumption of contaminated foods containing the *S. aureus* enterotoxins (SEs) (192). It has a rapid onset (2–8 h) with acute symptoms of nausea, vomiting, abdominal cramping and diarrhea (192, 193). Infants, elderly or immunocompromised patients are at high risk (193). The exotoxins are resistant to heat treatment and low pH allowing them to retain their activity in the digestive tract after ingestion (194). These toxins act on the vagus nerve in the abdominal viscera which triggers a signal to the vomiting center in the brain (198). Also, SEs are able penetrate the gut lining and activate local and systemic immune responses (199-201). All SEs genes are located on mobile genetic elements such as plasmids, prophages and pathogenicity islands (195-197). *S. aureus* contains 21 serologically distinct SE genes. The classical SE genes, ranging from SEA to SEE, are common cause of SFP. An additional 16 types of SE genes (*seg* to *sei*) were newly discovered after a genome study of *S. aureus* (172).

13.7. Reservoirs and Transmission

S. aureus is both a commensal organism and a pathogen capable of causing a vast array of disease. It colonizes the skin and mucosal surfaces of humans. The anterior nares of the nose are the preferred site of colonization. The bacterium can also be carried by the perineum, pharynx, and less frequently the

gastrointestinal tract, vagina and axillae (173). In the United States approximately 20% of individuals are nasally colonized persistently and 30% are colonized intermittently with high incidence among 6-7 year old children (165, 174). Colonization enables *S. aureus* transmission among individuals in both health care and community settings (165). The nasal carriage of *S. aureus*, is considered a major risk factor for patients undergoing surgery, hemodialysis, and patients with intravascular devices or HIV infection (175). Carriage of MRSA strains in the US population is less common but is continuously increasing. In the period between 2001 and 2004 the number of individuals carrying MRSA doubled from 0.8% to 1.5% (176).

It is hypothesized that this asymptomatic carriage, infected individuals as well as contaminated surfaces are prerequisites for the transmission of MRSA to healthy individuals in health-care facilities and in the community (43, 177). In hospital facilities healthcare workers and their hands can be an important source of MRSA dissemination (165, 178-180). Healthcare workers attend the needs of multiple patients in different areas of the hospital. Another important factor is the high rate of asymptomatic MRSA colonization among healthcare workers when compared to the general population. In the US, several recent studies have reported an average of 4.2% colonization for healthcare workers versus 1-2% for the general population (174, 181, 182).

Another important risk factor is the ability of MRSA to withstand environmental stress and survive for long periods of time on a variety of surfaces with different physicochemical properties such as steel, plastic, ceramic, soap,

wood, and vinyl. These can serve as a possible source for outbreaks hospitals and in the community (183-187). Shared surfaces or fomites such as whirlpools, razors, towels, and soap have been associated with CA-MRSA outbreaks among sport athletes (44, 188-190). The Centers for Disease Control and Prevention (CDC) summarizes the risk factors of MRSA transmission in the community as the 5 "C's": crowding, frequent skin-to-skin contact, compromised skin, contaminated items and surfaces, and lack of cleanliness. These risk factors are frequently present in community settings such as prisons, military barracks, college dormitories, daycare facilities, households, and contact sport facilities (191).

A recent report of the public transportation in Belgrade, Serbia, has shown high rates of MRSA strains present on handrails in trams or buses (192). Currently, there is a lack of knowledge regarding the survival and transmission of MRSA from fomites in an airplane cabin. Given the conditions on the board of a civil aircraft, gathering data on the subject would reduce the relative risk of infection for passengers during a commercial flight and would allow the development of adequate prevention strategies.

Summary

The majority of disease reports onboard of a civil aircraft are based on outbreak data. In most cases, these outbreaks originate as consequence of the spread of highly infectious airborne viral and bacterial pathogens. Usually, these incidents are a consequence of malfunctioning aircraft ventilation systems, the presence of asymptomatic passengers and the limited space in the cabin.

Although, it has been widely acknowledged that bacterial pathogens on surfaces are a risk factor in nosocomial and community associated infections, there are no studies evaluating their role as a source of disease in the aircraft cabin. As discussed in this chapter, *E. coli* O157:H7 and in particular MRSA are capable of colonizing asymptomatically healthy individuals. They can be spread by fomites, and survive on surfaces for months. Mass transportation vehicles such as the airplane, with high level of crowding and significant hand-to-fomite contact in the premises of the cabin, may represent an important site for transmission.

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Chapter 2

Survival and Transmission of *Escherichia coli* O157:H7 on Surfaces in the Airplane Cabin

Abstract

We determined the limits of survivability and transmissibility of *Escherichia coli* O157:H7 on three surfaces under typical aircraft cabin conditions, specifically 24 °C and < 18% air humidity. A concentration of 2.5 x 10^5 CFU/ml was inoculated on 1 x 1 cm^2 coupons of the rubber armrest, steel toilet handle and plastic tray table. PBS, simulated saliva and sweat were used as suspending media. Direct sonication was employed to detach the bacteria from the coupons. The standard plate method, direct real-time quantitative PCR (qPCR) and real-time quantitative PCR in combination with propidium monoazide (qPCR-PMA) were performed to quantify the persistence of the viable cells over time. The statistical analysis showed that qPCR-PMA was significantly more sensitive than the plate count ($p < 0.05$). *E. coli* O157:H7 survived the longest on arm rest, 96 hours, followed by tray table, 72h and steel toilet handle, 48h. The VBNC remained significantly longer on the surfaces in PBS, followed by simulated sweat and saliva ($p < 0.05$). A pigskin contact model was used to

determine the transfer rates from fomites to skin over time. The plastic tray had a significantly longer transmission rates compared to the other two surfaces ($p < 0.05$). Saliva and metal toilet handle had a detrimental effect on both survivability and transmissibility under the specified conditions ($p < 0.05$).The method used identifies the persistence of the VBNC state and provides data for the transmission of STEC from fomites on a civil aircraft. In addition, it allows monitoring of disinfection efficacy of contaminated surfaces on a civil aircraft.

Introduction

At present commercial airlines transport millions of passengers over vast distances in short time periods (1). The increased mobility of people has also enhanced the risk of disease outbreaks during the flight (2, 3). In the airplane cabin, travelers are at risk of infection because of the limited seat area, the high rates of fomite and human contact, the relatively low air humidity (< 18 %), and the immune state of some passengers (3-6). The potential spread of airborne life-threatening viral and bacterial pathogens within the aircraft has been documented in several articles (7-10). Currently, foodborne outbreaks due to contaminated in-flight catering is uncommon due to the rigorous safety standards, but it has been a matter of concern in the past (11).

Escherichia coli O157:H7 is the most prevalent enterohemorrhagic serovar found in the US, and has been responsible for multiple large foodborne outbreaks (12). In most cases, it causes hemorrhagic colitis and is often associated with severe systemic sequel such as the hemolytic uremic syndrome

(HUS). The shiga toxins (*Stxs*) produced by the bacteria act systemically on sensitive cells in the kidneys, brain, and other organs. Commonly, children less than 5 years old and the elderly are most susceptible to these severe complications (12).

The detection procedures to prevent possible outbreaks due to STEC contaminated food and water are based on culture, PCR and immunological assays (13-15). Other classical methods such as contact plates, sampling sponges, adhesive tapes, swabs, and ATP-based monitoring systems have been used to determine the level of microbial surface contamination and to assess the efficacy of the cleaning and disinfection procedures applied (16-18). However, these methods suffer from inadequate sensitivity, given the low infectious dose of *E. coli* O157:H7 and the ability of the bacterium to survive under stress conditions in a viable but non-culturable (VBNC) state (19-21). In this state bacteria maintain low metabolic activity and retain virulence, but grow poorly on routine microbiological media (20). Detection and quantification of VBNC by conventional PCR is of little value since PCR is unable to discriminate DNA from live or dead bacteria (22). DNA can persist intact after cell death and the presence of microorganisms may be overestimated. A new technique that uses the specificity and sensitivity of real-time quantitative PCR (PCR) based on the membrane integrity of bacteria, is now widely accepted as a method for distinguishing viable from dead cells (23). This method takes advantage of nucleic acid intercalating dyes such as ethidium monoazide (EMA), and propidium monoazide (PMA) (23). Both molecules contain an azide group and

selectively penetrate damaged membranes of dead cells and covalently link to DNA by photolysis under intense visible light, preventing the amplification of targeted sequences from dead cells (23). Another chemical, released by Biotecon Diagnostics under the name Reagent D, has been reported to act in an analogous mode to EMA and PMA. However, it has been demonstrated that EMA and Reagent D are not optimal in use of VBNC quantification (23, 24). Although both EMA and PMA intercalate the DNA of dead cells, PMA penetrates fewer viable cells and VBNC with undamaged membranes primarily due to its two positive charges (23). The real-time qPCR method combined with PMA (qPCR-PMA) has been successfully optimized and employed to detect VBNC cells of important foodborne bacterial pathogens such as *Salmonella*, *Listeria monocytogenes*, and *Campylobacter jejuni*, as well as *E. coli* O157:H7 in different types of food (25-27).

Although *E. coli* O157:H7 is primarily a foodborne pathogen, existing data suggest that outbreaks are occasionally caused by the presence of asymptomatic carriers shedding the bacteria for long periods of time in public locations such as child daycare centers, schools, and households (12, 28, 29). In such community settings, environmental surfaces or fomites can serve as vectors for transmission when contaminated with bacteria by direct contact with body fluids and contact with hands and mouth (30, 31). In addition, several reports have shown that STEC can survive on abiotic surfaces with different physicochemical properties under variety of environmental stressors for extended intervals of time which in turn can facilitate its transmission via aerosols or direct contact (28, 32-

37). Since the persistence of *E. coli* O157:H7 on fomites of shared use in community settings appears to be a risk contributing to outbreaks, we chose to determine the survival of this pathogen on common airplane cabin surfaces. Survival through adherence, biofilm formation and resistance to disinfectants of *E. coli* O157:H7 on surfaces in defined laboratory conditions are well known (32, 33, 37). However, these conditions do not represent the environment found in the airplane, in particular low air humidity (< 18 %) and relatively constant temperature (18-24ºC) (5). Therefore, we evaluated the survival of *E. coli* O157:H7 in three different media: phosphate buffered saline (PBS), simulated sweat, and saliva on three types of air cabin surfaces under typical aircraft cabin air conditions. We hypothesize that in addition to the low air humidity, survival and transmission rates of this pathogen depend on the physicochemical properties of each surface as well as the pH and contents of the simulated saliva and sweat. To address the possibility of some cells surviving in VBNC state, we used three methods, standard culture methods, real-time qPCR and qPCR-PMA to detect only viable cells over a period of time. To assess the secondary modes of transmission of STEC from contaminated surfaces in the airplane cabin, we used a modified pigskin model (38).

Airplane travel offers undeniable benefits for travelers and commerce. However, limited amount of data exist about bacterial pathogens that can survive for extended periods of time and possibly cause outbreaks during prolonged commercial flights. Given its low infectious dose, knowledge of the VBNC state of *E. coli* O157:H7 and its transmissibility to human skin in the aircraft cabin is of

great importance in order to provide data that can be used as microbial risk assessment to improve the rigor of cleaning and disinfection strategies.

Materials and methods

Bacteria and Inoculum Preparation

E. coli O157:H7 (ATCC 43894), an isolate originally from a patient with hemorrhagic colitis obtained from American Type Culture Collection (ATCC, Manassas, VA 20108) was used as a representative of hemorrhagic *E. coli* serotypes (39). This strain was initially stocked in 20% (*v*/*v*) glycerol at –80 °C, resuscitated in 2 ml BactoTM Tryptic Soy Broth (TSB; Beckton Dickinson and Co., Sparks, MD) and grown at 37°C until mid-log phase (OD_{600} = 0.5) and streaked on selective sorbitol MacConkey agar plate (Beckton Dickinson and Co., Sparks, MD) afterwards. One colony was recovered from the sorbitol MacConkey plate and cultured in 10 ml BactoTM Tryptic Soy Broth (TSB; Beckton Dickinson and Co., Sparks, MD) at 37 °C until late-log phase (OD_{600} = 1.2). Subsequently, the bacterial cells were harvested by centrifugation (6000 x rpm, 10 min) and the cell pellet was re-suspended in 10 ml of phosphate-buffered saline (1 x PBS, 137 mM sodium chloride, 2.7 mM potassium Chloride, 10 mM phosphate Buffer). The procedure was repeated three times to remove remaining nutrients. The final concentration was adjusted to $1x10^7$ CFU/ml, confirmed by spectrophotometry, serial dilution, and plating and divided into three tubes, 10 ml each. The PBS was removed from two of the tubes by centrifugation. In the first tube, the cells were mixed with 10 ml of simulated saliva (40). The solution was prepared using 1 L of distilled water and the following ingredients: 0.92 g/l xanthan gum, 0.85 g/l

sodium chloride, 1.2 g/l potassium chloride, 0.13 g/l calcium chloride, 0.05 g/l magnesium chloride, 0.13 g/l di-potassium hydrogen orthophosphate, and 0.35 g/l methyl p-hydroxybenzoate. The pH was adjusted to 6.7 using sodium hydroxide. The medium was sterilized by autoclaving. In the second tube, PBS was replaced by 10 ml of simulated sweat. The solution was prepared using 1L of distilled water, 20 g/L sodium chloride, 17.5 g/L ammonium chloride, 5 g/L acetic acid, and 15 g/L *d,l*-lactic acid (41). The pH was adjusted to 5.0 using sodium hydroxide, and the medium was sterilized by autoclaving.

Contamination of Air Cabin Surfaces

Air cabin surfaces were obtained from Delta Airlines (Delta Air Lines, Inc.). We used three surfaces for our survivability and transmissibility model: rubber armrest, stainless steel toilet handle and plastic tray table. Each surface was cut in coupons of 1 x 1 cm^2. The coupons were sterilized using a gamma (γ) - irradiating cobalt - 60 source at a γ-dose of 2.5 kGy (Leach Nuclear Science Center, Auburn University, AL). The three bacterial suspensions of *E. coli* O157:H7 were deposited in aliquots of 25 μl of $1x10^7$ CFU/ml on each surface coupon to achieve an approximate final concentration of 2.5 x 10^5 CFU/cm^2. A total of 40 coupons of each surface were inoculated with *E. coli* O157:H7 PBS. Twenty of the coupons were used for the survivability trial and the remaining twenty for the transmissibility assay. The same was done with the bacterium suspended in simulated sweat and saliva. Coupons of each surface inoculated with sterile PBS, saliva, and sweat were used as controls. All coupons were incubated

in an environmental chamber (Caron 6020, CARON Products & Services, Marietta, OH) at 24 °C with 18% air humidity.

Recovery of *E. coli* O157:H7 from Surfaces by Sonication

For the recovery of the surviving bacteria immediately after drying (60 min) and at intervals of 24 hours, two coupons per surface/liquid were placed aseptically in two separate sterile 50 ml polystyrene conical tubes (VWR International, Radnor, PA), each with 5 ml of sterile PBS. Afterwards, each coupon was sonicated by placing the probe of the sonicator 1 cm above the coupon inside PBS solution. Each coupon was sonicated for 20 sec at 2 W, 20 KHz (Sonicator Q700, Qsonica, LLC Newtown, CT) with a microtip (1.6 mm diameter) and then vortexed for 10 sec.

Culture Based Enumeration of Survivability

To assess the survivability over time, the obtained bacterial suspension from each sonicated surface coupon/ liquid were diluted 10-fold with sterilized PBS and 100 µl bacterial sample was evenly spread-plated in duplicate on TSA agar plate (Becton, Dickinson and Company, Sparks, MD). The same procedure was carried out for the control coupons. After incubation for 24 h at 37 °C, colony-forming units were counted for each plate, averaged and expressed as CFU/cm^2. Random colonies from spread plated coupons were streaked on to sorbitol MacConkey agar to confirm lack of possible cross contamination. *E. coli* O157:H7 was considered nonculturable when no CFU were present after 24 h of incubation. In addition, two aliquots of 500 µl from two sonicated coupons were

placed in two light-transparent 1.7 ml microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA), the first two for direct DNA extraction and the second two aliquots for DNA extraction after PMA treatment.

DNA Extraction for Real-time PCR

Direct DNA extraction was performed in parallel to spread plating at each time point for each set of two coupons per surface/liquid. Samples were centrifuged at 14,000 rpm for 10 min. The supernatant was removed from each tube and cell pellets were resuspended in 100 μl of PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems-ABI, Foster City, CA). Cell suspensions were vortexed for 5 s and placed for 10 min at 90 °C in a heat block. The samples were then centrifuged at 14. 000 rpm for 3 min and 50 μl DNA extracts were recovered from the supernatants and placed in new sterile microcentrifuge tubes and stored at 4 °C until PCR amplification.

PMA Treatment

The PMA (phenanthridium, 3-amino-8-azido-5-[3-(diethylmethylammonio) propyl]-6-phenyl dichloride) (Biotium, Inc.) treatment procedure was performed based on optimization previously described by Nocker and colleagues (23). An aliquot of 1.25 μl of 20 mM PMA was added to 500 μl suspensions, recovered after the sonication procedure, to a final concentration of 50 μM. Each light-transparent 1.7 ml microcentrifuge tube (Fisher Scientific, Pittsburgh, PA) was wrapped with aluminum foil. The mixture was incubated for 15 min in the dark, at room temperature with occasional mild vortexing. The aluminum foil was

removed, the tubes were horizontally placed on ice in a transparent container to prevent overheating and killing of the cells, and exposed to light for 5 minutes with a 650-W halogen light source (sealed beam lamp, FCW 120 V; GE Lighting, General Electric Co., Cleveland, OH) placed 20 cm away to allow cross-linking. The PMA treated cell suspensions were then harvested by centrifugation (10 000 rpm, for 10 min) and washed twice with DNAase / RNAase free water (Invitrogen, Carlsbad, CA) to remove trace amounts of PMA to avoid interference with DNA extraction and qPCR performance. DNA extraction followed the steps described in the previous section. The samples were stored at 4 °C until PCR amplification. A general representation of the procedure is summarized in Fig.1.

Real-time qPCR Parameters

The TaqMan approach was used to amplify the *eaeA* gene of *E. coli* O157:H7. The real-time qPCR was carried out in a 96-Well MicroAmp® Optical Reaction Plate (Applied Biosystems, Foster City, CA), using Mastercycler® ep realplex (Eppendorf, Hauppauge, NY) instrument and fluorescence measurements were analyzed with Realplex software (Eppendorf, Hauppauge, NY). Each reaction mixture contained 10 µl of template DNA, 2 x iQ™ Supermix (dNTPs, 6 mM $MgCl_2$, 50 U/ml iTaq™ DNA polymerase) from (Bio-Rad Laboratories, Hercules, CA), 0.5 µM forward and reverse primers, and 0.25 µM TaqMan probe. The sequences of the primers targeting the *eae*A gene (GenBank accession number AF081182) were designed according to Suo et al., (42), forward primer 5'-AACCACGGGAAATGATGGTC-3', reverse primer 5'-TTCCCTGATGCATCGACAGT-

3' and a TaqMan probe 5'-FAM-GGCTGAGGTTAAAGCGACTG-3'-BHQ1. The size of the PCR product was 294 bp. The Primer 3 software (v.0.4.0) was used to design the probe. An internal control consisted of EcoRI linearized pUC19 (New England Biolabs, Ipswich, MA). The primers, forward 5'-GCAGCCACTGGTAACAGGAT-3', reverse 5'-GCAGAGCGCAGATACCAAAT-3' and probe 5'-JOE-AGAGCGAGGTATGTAGGCGG-3'-BHQ1 probe targeting the pMB1 replicon rep) (Fricker et al. 2007) (43). Primers were used at a concentration of 0.25 μM and the probe at 0.2 μM. The size of the PCR product was 118 bp. The optimal concentration of linearized pUC19 used was 104 copies/PCR and the parameters were previously optimized by Fratamico and DebRoy, 2010 (44). DNAase/RNAase free water (Invitrogen, Carlsbad, CA) was added to the mix to a final reaction volume of 50 μl. The PCR cycling conditions were as follows: initial denaturation 95 °C for 10 min; 40 cycles at 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 30 s. In every qPCR analysis, the *E. coli* O157:H7 standard for absolute quantification was included in duplicate. In addition, a nontemplate control containing 10 μl DNAase/RNAase free water (Invitrogen, Carlsbad, CA) and negative control (5 ng Staphylococcus aureus) were included. Two coupons per surface, inoculation media and time point were used for PCR analysis in triplicate. The Realplex software (Eppendorf, Hauppauge, NY) provided the C_T values that were converted to log10-transformed cell number per 1 cm^2 surface using the standard curve.

PMA Inhibition of qPCR signal from Dead Cells

To obtain *E. coli* O157:H7 dead cells, an equal portion of each serial dilution, 1 x 10^2, 1 x 10^3, 1 x 10^4, 1 x 10^5, and 1 x 10^6 CFU/ml, were heat-treated at 80 °C for 10 min. The absence of cell viability was confirmed by plating. The samples were analyzed in duplicate by real-time PCR to confirm the signal reduction capacity of PMA sample treatment.

Standard Curve for Absolute Quantitation

A 10-fold serial dilution starting from 1 x 10^2 to 1 x 10^6 CFU was prepared in 1 ml PBS from an overnight culture of *E. coli* O157:H7. Bacteria were enumerated by spread plating in triplicate. Overall, two independent experiments with triplicates were used to generate the standard curve. The *Ct* values obtained with the qPCR were plotted against the number of CFU (log CFU) in the standards.

Preparation of Pigskin

Pigskin was obtained from a freshly slaughtered pig (Dean Sausage Company, Inc., Attalla Al). The pigskin was washed, shaved, and the fat tissue was removed. Subsequently the skin was cut in skin swatches of 1 x 1 cm^2. To eliminate the resident bacteria, we modified the protocol of Maish et al. (45) and instead of incubating the skin in 70% ethanol for 5 min, the pigskin swatches were placed in 200 ml Pyrex bottles (Corning Incorporated, U.S) and sterilized using a gamma (γ) - irradiating cobalt - 60 source at a γ-dose of 25 kGy (Leach Nuclear Science Center, Auburn University, AL).

Transmissibility from Surfaces to Pigskin

Immediately after drying, two coupons per surface/media were retrieved and placed aseptically in a sterile 100 x 25 mm Petri dish (VWR International, Radnor, PA). A separate pigskin swatch was pressed against each individual surface coupon for 3 sec. Each pigskin swatch was then placed into separate sterile 50 ml polystyrene conical tube (VWR International, Radnor, PA) containing 1 ml of PBS and vortexed for 30 sec. Serial dilutions of the resulting solution were prepared, and 100 µl was plated onto TSA agar plate before incubating for 24 hours at 37 °C. The functionality of the pigskin transmission model was assessed by inoculating 2.5 x 10^5 CFU/ml of *E. coli* O157:H7 suspended in PBS, saliva, and sweat directly onto pigskin. As a negative control, we used the resultant PBS suspension of vortexed sterile pigskin swatches pressed to surface coupons inoculated with sterile PBS, saliva, and sweat without prior inoculation with *E. coli* O157:H7.

Statistical Analysis

The survival (plate counts and qPCR) and transmission trials were repeated twice (to get a total of 4 replicas per time point). A general linear mixed effects model with repeated measures and a post-hoc Tukey test were used to compare the $\log_{10}$-transformed data obtained by the direct qPCR, qPCR-PMA procedures and the standard plate method. Time, quantification method, surface type and inoculation media were included as fixed effects. Time by quantification method, time by surface and time by type of media interaction were also included in the analysis. The transmissibility from a surface to the pigskin was analyzed by

linear regression using the standard plate count data only. It compared the average survivability using the absolute number of *E. coli* O157:H7 per time and surface to the average transmitted bacteria recovered from pigskin from the corresponding surface and time point. Differences between means were considered significant at ($p < 0.05$). The *STATISTICA* 10 Software for Windows (StatSoft, Inc. Tulsa, OK) was used to determine the statistical significance.

Results

Standard Curve for Absolute Quantification

The standard curve obtained from DNA extracted from 10-fold dilutions of *E. coli* O157:H7 showed an average efficiency of 97%, computed from the slope of the linear relationship between $\log_{10}$ transformed number of CFU per milliliter and the C_T value ($R^2 = 0.999$) (Fig. 2). The method was linear over a range of 1×10^2 to 1×10^6 CFU /ml and the limit of quantification was 1×10^2 CFU/ml. Given the volume reduction during DNA extraction, this corresponds to 10 CFU/PCR. From the standard curve, the concentration of *E. coli* O157:H7 bacteria retrieved by sonication from each of the air cabin surface coupon was expressed by the equation $\log \text{CFU} = (C_T - 40.33) / -3.441$.

PMA Inhibition of qPCR Signal from Dead Cells

The C_T values obtained by qPCR of the 10-fold dilution series of heat-treated *E. coli* O157:H7 with and without PMA sample treatment are shown in Fig. 3. No viable *E. coli* O157:H7 bacteria were detected by the standard plate method after the heat treatment. The signal reduction for the PMA-treated

samples was 100%, respectively; no signals were obtained from PMA-treated samples by qPCR.

Survivability of *E. coli* O157:H7 Bacteria on Air Cabin Surfaces

The survivability *E. coli* O157:H7 was determined by direct qPCR, qPCR-PMA and plate counts. The data obtained were $\log_{10}$ transformed using the general linear mixed model procedure. The analysis with post-hoc Tukey test indicates bacterial recovery from all surfaces decreased over time regardless of the quantification method. The sensitivity of each method was in the following order: qPCR > qPCR-PMA > standard plate count ($p < 0.05$) (Table 1). The total number of *E. coli* O157:H7 cells quantified by direct qPCR remained constant, 4 log approximately, for the duration of the experiment, regardless of the type of media used (Fig. 4). The number of viable cells detected using qPCR-PMA remained above the detection limit for ≥ 96 hours on all surfaces when deposited in PBS, and the cell counts were significantly higher for all time points than the estimated by the standard plate count method ($p < 0.05$). The qPCR-PMA method was also more sensitive in detecting cells in the VBNC state retrieved from surfaces deposited in saliva and sweat ($p < 0.05$). Surface samples recovered from coupons inoculated with saliva did not yield any plate counts at any time points, except from armrest at time 0 h. In contrast, viable bacteria were recovered from these surfaces for ≥ 48 hours by qPCR-PMA and were above the minimum infectious dose of *E. coli* O157:H7 (Fig. 4). No significant difference between qPCR-PMA and standard plate counts were observed when bacteria were deposited in sweat and recovered from steel toilet handle (at 0 and 24 hours) and

plastic tray table (at 24 hours) ($p < 0.05$). Viable cells remained higher for the rest of the surface samples in sweat for ≥ 72 hours (Table 1). Depending on the medium used, the results with the qPCR-PMA method indicate that viable O157:H7 persisted on the surfaces in the following order PBS, ≥ 96 hours > sweat, ≥ 72 hours > saliva, ≥ 48 hours ($p < 0.05$). *E. coli* O157:H7 survived the longest on the armrest (≥ 96 hours), followed by tray table (≥ 72 hours) and steel toilet handle (≥ 48 hours) (Fig. 4).

Transmission from Surfaces to Pigskin

In order to determine the functionality of our transmission model, we inoculated 2.5 x 10^5 CFU/ml of *E. coli* O157:H7 suspended in PBS, saliva and sweat directly onto pigskin. We recovered 1.7 x 10^5 CFU/ml from PBS, 1.3 x 10^5 CFU/ml from saliva and 1.5 x 10^5 CFU/ml from sweat: 68%, 52%, and 60% of the initial inoculum, respectively. The negative controls inoculated with sterile PBS, saliva, and sweat and pressed with sterile pigskin showed no plate counts with *E. coli* O157:H7 or pigskin resident bacteria at any time point. The percentage rates of bacterial transmission to pigskin according to their survivability are summarized in Table 3. Independently of the surface type, no significant differences in transmission rates were observed between PBS and sweat ($p < 0.05$). However, both media had higher rates than saliva ($p < 0.05$). As for the survivability, saliva had a significantly detrimental effect on the transmissibility of O157:H7. The plastic tray table had significantly longer transmission rates in PBS and sweat than armrest and steel toilet handle coupons in the same media, ≥ 96 hours ($p < 0.05$). In contrast, the steel toilet handle

material had the lowest counts recovered from pigskin, regardless of the medium used for the initial inoculum ($p < 0.05$).

Discussion

The current globalization trends show a huge increase in the numbers of people travelling abroad. Millions of passengers are taking advantage of affordable airlines and cover vast distances in short times (1). This increased mobility of people, often to remote areas of the world where certain pathogens are endemic, has increased the potential for outbreaks and transmission of infectious diseases during and after a commercial air flight (16, (2). Infections due to airborne life-threatening viral and bacterial pathogens within the aircraft have been reported in the past (7-10). In the current study, we evaluated *E. coli* O157:H7 as a pathogen that can persist in VBNC state and cause infections in the airplane cabin via direct transmission through fomites.

We employed gamma irradiation as a method for sterilization of the surfaces. This method allowed us to preserve the physicochemical properties of the fomites using a dose of 25 kGy, much higher than the suggested in reports for killing *E. coli* (46). To detach the bacteria from each surface coupon, we used the direct sonication method. We chose this approach because the swabbing technique has been shown to have lower efficiency when sampling dry surfaces (47).

To quantify the survival of *E. coli* O157:H7 we used two methods, standard plate counts and qPCR. The qPCR technology offers undeniable advantages regarding bacterial pathogen quantification and detection from variety

of sample sources. However, a major drawback of this approach is that it operates on a DNA level and cannot distinguish between DNA from viable bacterial cells and that from dead bacterial cells. This limitation can lead to over estimation of the number of viable cells and often delivers false positive results (22). Our results also support the lack of specificity of direct qPCR to distinguish dead and viable cells. We demonstrated that the qPCR-PMA approach can effectively distinguish between viable and dead cells when samples were treated with PMA prior to the DNA extraction. The specificity of the qPCR-PMA assay method was 100%, given the target-specific DNA TaqMan DNA probe-based and primers were used. In addition, the specificity and stability of the assay were aided by the internal amplification control; thus, reducing the possibility of false negatives due to the presence of inhibitors. The high amplification efficiency of the assay also supports the suitability of the assay for quantitative analysis. In terms of sensitivity, our results also indicate this method is significantly more sensitive than the culture-based method. Besides being labor-intensive and time consuming, the culture-based quantification will not detect the VBNC fraction of a given *E. coli* O157:H7 population. The low infectious dose of *E. coli* O157:H7 and the ability of the organism to enter in VBNC state under stress makes colony-based quantification unsuitable for developing adequate surface cleaning strategies (19-21).

Although, the qPCR-PMA approach has also been credited by other reports as a specific method for detecting only viable bacteria, an obvious limitation of the technique is that it relies exclusively on membrane integrity of

the VBNC cells (23). However, viability can also be defined by the ability of the bacterium to metabolize compounds and maintain a proton gradient between the inner and outer parts of the cell. The permeability of the bacterial cell might not be a straightforward reflection of whether the cell is viable or dead. It is rational to consider that PMA may also bind to DNA from injured cells that can repair their damaged membranes and appear VBNC right after the initial time point (0 h).

Our data show bacterial survival and transmission are influenced by three parameters: time, physicochemical properties of the fomites and the type of medium used. In general, bacterial densities decreased over time. Nevertheless, such data may be considered false negative results, since cell and DNA losses could have occurred during sonication, sample processing and dilution while preparing the PCR reaction. Therefore, a drawback of our qPCR-PMA was the quantification limit of 100 CFU/ml, higher than the infectious dose of *E. coli* O157:H7 reported in some studies (19).

Compared to the survivability data obtained in this study, other reports have shown longer survival rates of *E. coli* O157:H7 on abiotic surfaces (32, 33, 37). This disparity can be attributed to the higher initial inoculum, the higher relative humidity and lower temperatures used in these studies. Our data indicate survival was more prolonged on the rubber armrest, followed by the plastic tray table and shortest on the steel toilet handle. We speculate that because the rubber armrest is a porous fomite it offers bigger surface area for attachment, shielding the cells from dehydration and other stressors, and allowing longer persistence.

The results of our study also indicate higher survivability on hydrophobic (rubber and plastic) versus hydrophilic surfaces (metal toilet handle). Previous research reports have shown *E. coli* can form biofilms more rapidly on hydrophobic surface than on hydrophilic substrates (48). We are unaware whether this surface had trace amounts of copper that can trigger membrane lipid peroxidation which in turn damages bacterial membrane integrity.

Our survivability trials also indicate that *E. coli* O157:H7 survival is enhanced in PBS, and is more sustained in simulated sweat than in saliva. The presence of organic acids such lactic and acetic acid in the simulated sweat recipe have a negative effect on survivability when compared to PBS. Lactic and acetic acids can penetrate through the cell wall and cytoplasmic membrane and reduce the intracellular pH, disrupting the proton motive force. However, our results indicate survivability was significantly longer in sweat than in saliva. Past reports have shown that the pathogen may survive decontamination treatments of meat with lactic or acetic acid, suggesting that *E. coli* O157:H7 can adapt to the presence of organic acids (49).

The persistence of *E. coli* O157:H7 was also negatively affected when suspended in simulated saliva compared to PBS and sweat. We hypothesize that the presence of methyl p-hydroxybenzoate (methylparaben) in the formulation has a negative effect on *E. coli* O157:H7 survivability. Parabens cause loss of the membrane semi-permeability and energy uncoupling by inhibiting the uptake of metabolites and are widely used as antibacterial preservatives in pharmaceutical, cosmetic, and food products (50).

To assess the transmissibility of *E. coli* O157:H7 from fomites to skin, we modified the pigskin sterilization method described by Maish et al., 2007 (42). Gamma sterilization allows the elimination of the resident bacteria from the skin without causing alterations to the tissue. It has been routinely used in the past for sterilization of skin, bone, and other allograft tissues (43, 49). To recreate a mode of contact for transmission, we pressed pigskin swatches for 3 sec to the contaminated coupons. It is unclear whether this type of contact would offer enough time and pressure for the efficient transmission to the skin. Earlier reports have indicated that friction can increase the level of *S. aureus* transfer from fomites to skin by up to five-fold (50).

According to our pigskin model, the nonporous plastic tray table had significantly longer transmissibility rate than rubber armrest and metal toilet handle materials. The flat homogeneous plastic surface allows a more uniform contact with the pigskin compared to the porous rubber armrest. Saliva had a negative effect on transmissibility from the fomites tested; bacterial counts were recovered only at time 0 h from the armrest coupons. We speculate that the presence of xanthan gum in the formulation of the simulated saliva fixed the bacteria and prevented the optimal contact with the pigskin reflected in the absence of transmissibility. Besides as a saliva substitute, this polysaccharide is often used as a microencapsulation material of probiotic bacteria for their long term preservation in harsh environmental conditions (45). Overall, our data indicate that bacterial survival was positively correlated with transmission to the pigskin over time.

Conclusion

This study evaluated *E. coli* O157:H7 survival and transmission from three surfaces present in the airplane cabin. The employed methods, especially the qPCR-PMA identified the persistence of the VBNC state over time and provided data for the transmission of O157:H7 serovar from fomites under typical aircraft cabin conditions. We believe the collected data would aid to develop more efficient and cost-effective cleaning and disinfection procedures for the airplane cabin surfaces.

Figures and Tables

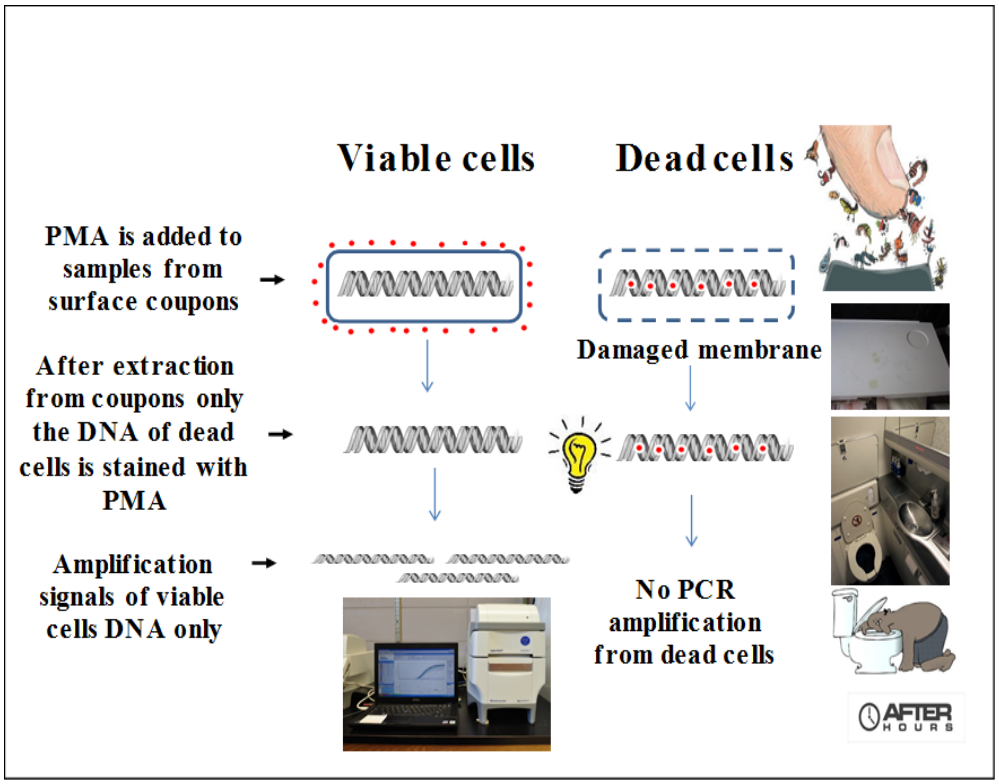

Figure 1. Schematic representation of the qPCR-PMA method

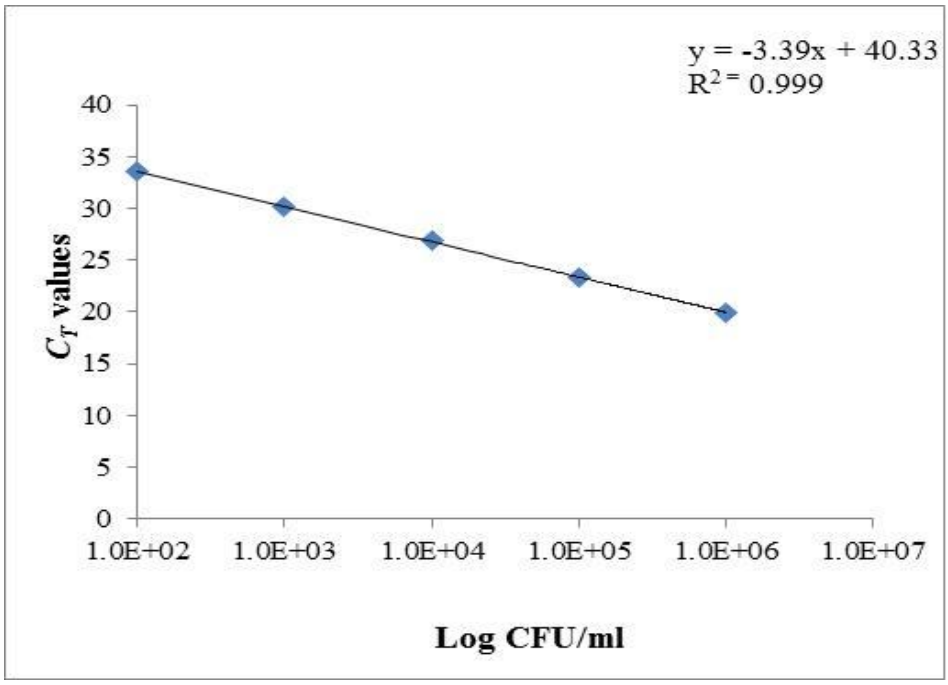

Figure 2. Standard curve for quantification of *E. coli* O157:H7 by qPCR

The curve was prepared with 10-fold serial dilutions ranging from $1x10^2$ to $1x10^6$ CFU/ml generated by TaqMan qPCR. The average value of the linear coefficient of regression (R^2) obtained in two independent experiments with triplicates is indicated.

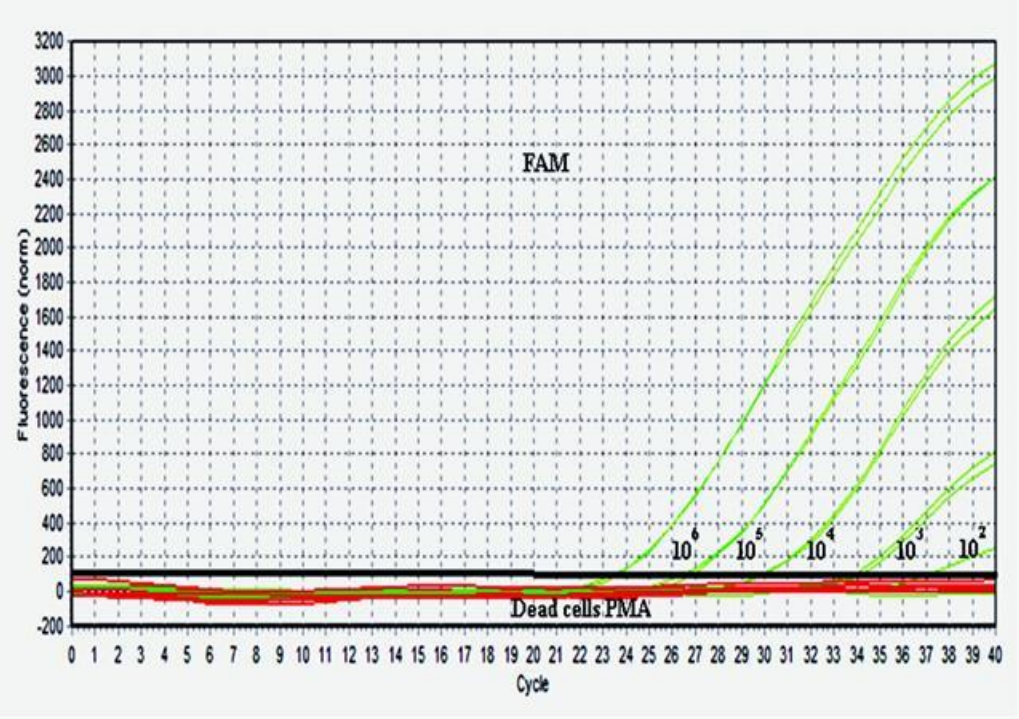

Figure 3. qPCR-PMA amplification of dead *E. coli* O157:H7 cells

The C_T values obtained from 10-fold dilution series of *E. coli* O157:H7 heat-killed cells, with and without PMA treatment. Green lines represent DNA amplification of dead cells without PMA treatment. The curves below the threshold line (red lines) represent a duplicate analysis of samples that received PMA treatment prior the PCR analysis.

		PBS				Saliva				Sweat			
Surface	Time hour	Plate Count $\log_{10}$	qPCR	PMA	Significance	Plate Count $\log_{10}$	qPCR	PMA	Significance	Plate Count $\log_{10}$	qPCR	PMA	Significance
Arm rest	0	3.01	4.19	3.33	b, c, d	2.03	4.36	3.45	a NS b, d (0.066)	2.54	4.54	3.04	b, c, d
	24	2.36	4.10	3.06	b, c, d	.00	4.14	3.34	b, c, d	2.14	4.48	2.68	b, c, d
	48	2.01	3.84	2.87	b, c, d	.00	4.09	2.17	b, c, d	1.47	4.43	2.69	b, c, d
	72	1.87	3.75	2.60	b, c, d	.00	3.84	.00	a NS b, c (1.000)	.00	4.20	1.70	b, c, d
	96	1.09	3.30	2.45	a NS b, c (0.058)	.00	.00	.00	a NS b, c (1.000)	.00	.00	.00	a NS b, c (1.000)
Toilet handle	0	2.63	4.52	3.17	b, c, d	.00	4.63	1.93	b, c, d	2.58	4.80	2.91	a NS b, c (0.494)
	24	1.76	4.29	2.67	b, c, d	.00	4.63	2.05	b, c, d	2.28	4.75	2.61	a NS b, c (0.594)
	48	1.18	4.18	2.58	b, c, d	.00	4.63	1.70	b, c, d	.00	4.68	2.05	b, c, d
	72	.00	4.14	2.58	b, c, d	.00	3.81	.00	a NS b, c (1.000)	.00	4.43	1.22	b, c, d
	96	.00	3.61	2.28	b, c, d	.00	.00	.00	a NS	.00	.00	.00	N/A
Tray table	0	2.34	4.45	2.93	b, c, d	.00	4.48	2.18	b, c, d	2.59	4.67	2.80	b, c, d
	24	1.70	3.84	2.57	b, c, d	.00	4.22	1.61	b, c, d	2.42	4.62	2.48	a NS b, c (0.941)
	48	1.50	3.78	2.53	b, c, d	.00	4.12	.00	a NS b, c (1.000)	.00	4.57	1.89	b, c, d
	72	1.09	3.57	2.34	b, c, d	.00	3.95	.00	a NS b, c (1.000)	.00	4.51	.00	a NS b, c (1.000)
	96	.00	3.48	2.28	b, c, d	.00	.00	.00	a NS b, c (1.000)	.00	.00	.00	N/A

Table 1. Statistical analysis of O157:H7 survival on surfaces over time

Significant differences were defined by general linear mixed effects model with repeated measures after Post-hoc Tukey test. [a] Non-significant. [b] Significant difference between plate counting and qPCR. [c] Significant difference between QPCR and qPCR-PMA. [d] Significant difference between plate counting and qPCR-PMA.

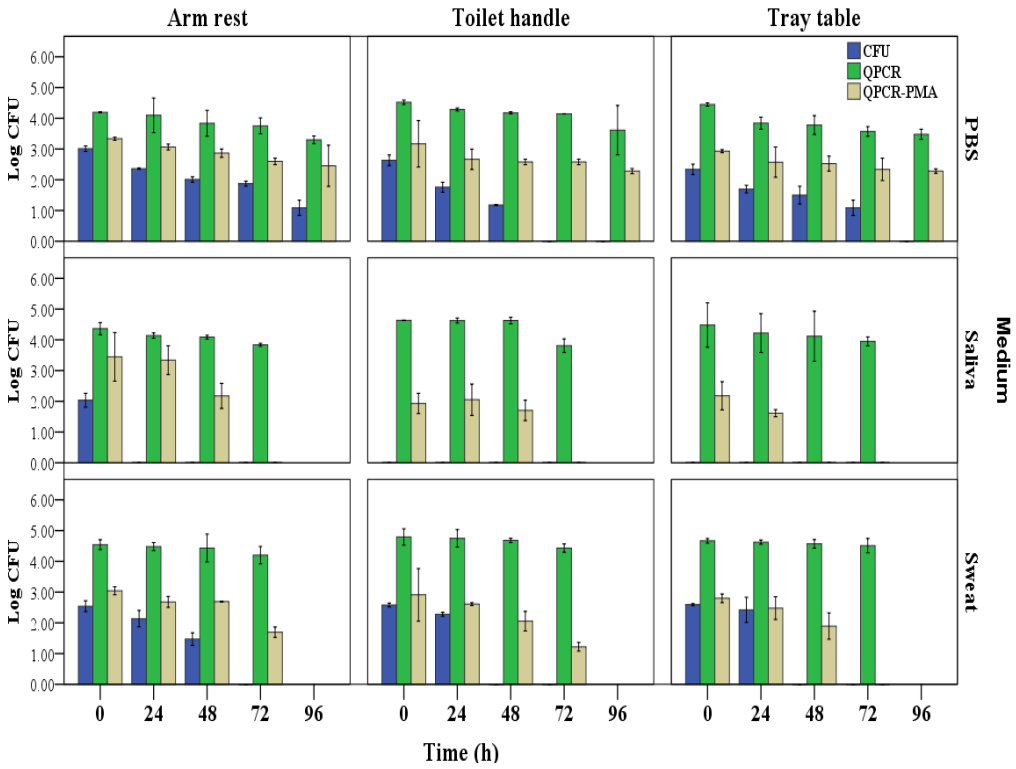

Figure 4. Quantification of *E. coli* O157:H7 survival by three different methods

Detection of *E. coli* O157:H7 ATCC 43894 viable but non-culturable cells on three air cabin fomites in three different suspending media assayed by direct plating (blue), direct real-time qPCR (green) and qPCR combined with PMA (beige). The bars represent standard deviations from two independent biological replicas and three PCR replicas.

Surface	Time (h)	Survival PBS	Skin PBS	Transmission	Survival Saliva	Skin Saliva	Transmission	Survival Sweat	Skin Sweat	Transmission
Arm rest	0	1.0E+03	3.8E+02	37%	1.10E+02	9.5E+01	86%	3.5E+02	1.8E+02	51%
	24	2.3E+02	5.5E+01	24%	0	0	0%	2.3E+02	5.5E+01	24%
	48	1.0E+02	2.0E+01	20%	0	0	0%	1.0E+02	0.0E+00	0%
	72	7.5E+01	0.0E+00	0%	0	0	0%	0.0E+00	0.0E+00	0%
Toilet handle	0	4.4E+02	2.5E+02	56%	0	0	0%	3.8E+02	2.9E+02	76%
	24	5.8E+01	1.5E+01	26%	0	0	0%	1.9E+02	5.5E+01	29%
	48	1.5E+01	0.0E+00	0%	0	0	0%	0.0E+00	0.0E+00	0%
	72	0.0E+00	0.0E+00	0%	0	0	0%	0.0E+00	0.0E+00	0%
Tray table	0	2.2E+02	2.0E+02	89%	0	0	0%	3.8E+02	3.3E+02	87%
	24	5.0E+01	3.5E+01	70%	0	0	0%	2.8E+02	2.2E+02	77%
	48	3.3E+01	2.0E+01	61%	0	0	0%	0.0E+00	5.5E+01	> 100%
	72	1.3E+01	1.0E+01	80%	0	0	0%	0.0E+00	1.0E+01	> 100%

Table 2. Survival and transmission of *E. coli* O157:H7 from surfaces

Survival and transmission rates of *E. coli* O157:H7 were measured at 0 hours (right after drying the inoculum), 24 h, 48 h, and 72 h after inoculation. Bacterial survivability was assessed by standard plate counts. Transmissibility was assessed using the pigskin model, vortexing, and standard plate counts and expressed as percentage of transmission given the survivability counts. The following formula was used:

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

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Chapter 3

Assessment of Survival and Transmission of Community-Associated Methicillin-Resistant *Staphylococcus aureus* on Common Surfaces in the Airplane Cabin

Abstract

We determined the limits of survivability and transmissibility of methicillin-resistant *Staphylococcus aureus* (MRSA) on fomites commonly found in a civil aircraft. A concentration of 2.5 x 10^5 CFU/ml was inoculated onto coupons (1 x 1 cm^2) of six different surfaces. Phosphate buffered saline (PBS), simulated human saliva and sweat were used as suspending media. Direct sonication was employed to detach the bacteria from the coupons and standard plate counts were performed right after drying and then intermittently every 24 hours over a period of seven days with inoculated coupons exposed to 24 °C and 18% humidity. A pigskin contact model was used to determine the transfer rates from fomites to skin over time. A general linear mixed effects model with repeated measures was used to analyze the data. Recovery rates decreased over time but more rapidly for non-porous fomites (metal toilet handle, plastic tray table and plastic window shade) as compared to porous fomites (armrest, leather seat, and pocket cloth) regardless the type of media used ($p < 0.05$). Independently of the surface, MRSA remained culturable longer in PBS than in

saliva and sweat ($p < 0.05$). Survivability was significantly longest on pocket cloth, up to 7 days and shortest steel toilet handle ($p < 0.05$). The pigskin model data indicates that the nonporous surfaces had significantly longer transmissibility than the rest of the studied surfaces in PBS and sweat ($p < 0.05$). However, porous fomites had significantly longer transmission rates in saliva compared to the nonporous surfaces in the same media ($p < 0.05$).

Introduction

Hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) is among the leading nosocomial pathogens in the United States (1). It is a frequent cause of skin and soft tissue infections (SSTIs), endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, foreign-body infections, and sepsis (1). Until recently, these infections were considered limited to the hospital environment, and patients treated at these facilities were among the population at risk. As a consequence, the study of MRSA outbreaks has concentrated primarily on health care facilities and persons such as elderly in nursing homes, the immunocompromised, and drug users who are at an obvious risk (2-4). In the past two decades incidents of MRSA disease have increased drastically as a result of the emergence of community-associated MRSA (CA-MRSA) strains, and the disease has spread to healthy individuals with no prior history of hospitalization (5). Skin and soft-tissue infections are the most common type of CA-MRSA infections (6). However, acute infections, such as necrotizing pneumonia, necrotizing fasciitis, and sepsis are not uncommon (7-9). In addition

to the capacity to cause disease, an alarming characteristic of this pathogen is its ability to asymptomatically colonize healthy individuals who are called "asymptomatic carriers". Carriage of MRSA strains in the US population in a period between 2001 and 2004 doubled from 0.8% to 1.5% (10). The most common sites of colonization are the anterior nares, although frequent sites of colonization are also the throat, the perineum, and the axilla (11). It is hypothesized that this asymptomatic carriage and contaminated surfaces are prerequisites for the transmission of CA-MRSA to healthy individuals in the community (12). Numerous studies have confirmed that MRSA has the ability to withstand environmental stress and survive for long periods of time on a variety of surfaces with different physicochemical properties such as steel, plastic, ceramic, soap, wood, and vinyl. These can serve as a possible source for outbreaks in the community (13-17). Shared surfaces or fomites such as whirlpools, razors, towels, and soap have been associated with CA-MRSA outbreaks among sport athletes (18-21).

The Centers for Disease Control and Prevention (CDC) summarizes the risk factors of MRSA transmission in the community as the 5 "C's": crowding, frequent skin-to-skin contact, compromised skin, contaminated items and surfaces, and lack of cleanliness. These risk factors are frequently present in community settings such as prisons, military barracks, college dormitories, daycare facilities, households, and contact sport facilities (22). The list of community settings at risk is permanently expanding. A recent surveillance study

of the public transportation in Belgrade, Serbia, have shown high rates of MRSA strains present on handrails in trams or buses (23).

In the context of airplane traveling, in particular the conditions in the airplane cabin, these factors are met and may trigger CA-MRSA outbreaks during a flight. In the airplane cabin, travelers can be at risk for MRSA infection because of the restricted seat space, the high rates of fomite and human contact, the relatively low air humidity (< 18%), the immune state of some passengers, and the possible presence of asymptomatic carriers of pathogens (24, 25). Given the limited number of lavatories, poor hand hygiene can also be among the risk factors. As noted in some studies, hand hygiene can reduce the incidence of MRSA transmission (26).

Since the survival and transmission (CA-MRSA) from fomites appears to play an important role in CA-MRSA outbreaks, the goal of our study was to determine the limits for the survival and transmission of CA-MRSA strain on common surfaces found in a civil aircraft under typical air cabin conditions. We hypothesized that survival and transmission rates differ depending on the physicochemical properties of each surface. In addition, we speculated that the presence of simulated saliva and sweat used to mimic human body fluids may prolong the survivability of MRSA. To study the transmissibility of bacteria from fomites to human skin, we developed a pigskin model. To the best of our knowledge the current study is the first attempt to study the survivability and transmission of MRSA from fomites present in the airplane cabin. We believe the current data would extend our knowledge of the relative risk of infection for

passengers during a commercial airplane flight, and would help to develop new sanitation and disinfection procedures for the airplane cabin surfaces.

Materials and methods

Bacteria and inoculum preparation

A community-acquired strain of MRSA MW2 (*Staphylococcus aureus* (ATCC BAA1707) was obtained from American Type Culture Collection (ATCC, Manassas, VA 20108) (isolated in 1998 in a CA-MRSA outbreak in North Dakota, US) (27). This strain was initially stocked in 20% (*v*/*v*) glycerol at –80 °C, resuscitated in 2 ml BactoTM Tryptic Soy Broth (TSB; Beckton Dickinson and Co., Sparks, MD) at 37 °C until mid-log phase (OD_{600} = 0.5) and streaked on a selective MRSA chromagar plate (Beckton Dickinson and Co., Sparks, MD) afterwards. One colony was recovered from the MRSA chromagar plate and cultured in 10 ml BactoTM Tryptic Soy Broth (TSB; Beckton Dickinson and Co., Sparks, MD) at 37 °C until late-log phase (OD_{600} = 1.2). Subsequently, the bacterial cells were harvested by centrifugation (6000 x rpm, 10 min) and the cell pellet was re-suspended in phosphate-buffered saline (1 x PBS, 137 mM sodium chloride, 2.7 mM potassium Chloride, 10 mM phosphate Buffer). The procedure was repeated three times to remove remaining nutrients. The final concentration was adjusted to $1x10^7$ CFU/ml, confirmed by spectrophotometry, serial dilution, and plating onto Trypticase Soy^{TM} Agar (TSA; Beckton Dickinson and Co., Sparks, MD) and divided into three tubes, 10 ml each. The PBS was removed from two of the tubes by centrifuging. In the first tube the cells were mixed with 10 ml of simulated saliva (28). The solution was prepared using 1 L of

distilled water and the following ingredients: 0.92 g/l xanthan gum, 0.85 g/l sodium chloride, 1.2 g/l potassium chloride, 0.13 g/l calcium chloride, 0.05 g/l magnesium chloride, 0.13 g/l di-potassium hydrogen orthophosphate, and 0.35 g/l methyl p-hydroxybenzoate. The pH was adjusted to 8.0 using sodium hydroxide. In the second tube PBS was replaced by 10 ml of artificial sweat. The solution was prepared using 1L of distilled water, 20 g/L sodium chloride, 17.5 g/L ammonium chloride, 5 g/L acetic acid, and 15 g/L *d,l*-lactic acid (29). The pH was adjusted to 5.0 using sodium hydroxide.

Artificial Contamination of Air Cabin Surfaces

Air cabin surfaces were obtained from Delta Airlines (Delta Air Lines, Inc.). We used six surfaces for our survivability and transmissibility model: rubber armrest, stainless steel toilet handle, plastic tray table, plastic window shade, leather seat, and seat pocket cloth (Fig 5.). The surfaces were cut in coupons of 1 x 1 cm^2. These surfaces were selected based on information of the presumed role of certain fomites in published reports of CA-MRSA outbreaks (16, 19, 30). The coupons were sterilized using a gamma (γ) - irradiating cobalt - 60 source at a γ-dose of 25 kGy (Leach Nuclear Science Center, Auburn University, AL), as used by Clavero et al., 1994. The three bacterial suspensions of MRSA were deposited in aliquots of 25 µl of $1x10^7$ CFU/ml on each surface coupon to achieve an approximate final concentration of 2.5 x 10^5 CFU/cm^2. A total of 28 coupons of each surface were inoculated with MRSA MW2 in PBS. The same was done with the bacterium suspended in sweat and saliva. Coupons of

each surface inoculated with sterile PBS, saliva, and sweat were used as controls. All coupons were incubated in an environmental chamber (Caron 6020, CARON Products & Services, OH) at 24 °C with 18% relative air humidity. To ensure consistency in terms of survivability and transmission, the experiment was repeated the following week with a new set of 28 coupons per surface/liquid (PBS, saliva, sweat).

Preparation of Pigskin

Pigskin was obtained from a freshly slaughtered pig (Dean Sausage Company, Inc., Attalla Al). The pigskin was washed, shaved, and the fat tissue was removed. Subsequently the skin was cut in skin swatches of 1 x 1 cm^2. To eliminate the resident bacteria we modified the protocol of Maish et al., 2007 (31) and instead of incubating the skin in 70% ethanol for 5 min, the pigskin swatches were placed in 200 ml Pyrex bottles (Corning Incorporated, U.S) and sterilized using a gamma (γ) - irradiating cobalt - 60 source at a γ-dose of 25 kGy (Leach Nuclear Science Center, Auburn University, AL), as suggested by Rooney et al., 2008 (32).

Recovery of MRSA from Air Cabin Surfaces by Sonication

To recover the surviving bacteria right after drying (30 min) and then every 24 h, two coupons per surface/liquid were placed aseptically in two separate sterile 50 ml polystyrene conical tubes (VWR International, Radnor, PA) each containing 5 ml of sterile PBS. Afterwards each coupon was sonicated by placing the probe of the sonicator 1 cm above the coupon inside PBS solution. Each

coupon was sonicated for 20 sec at 2 W, 20 KHz (Sonicator Q700, Qsonica, LLC Newtown, CT) as reported by Juhna et al., 2007 (33) and then vortexed for 10 sec.

Culture Based Enumeration of the Survivability over Time

To assess the survivability over time, the obtained bacterial suspension from each sonicated surface coupon/liquid was diluted 10-fold with sterilized PBS and a 100 µl bacterial sample was evenly spread plated in replicas of two on TSA agar plates (Becton, Dickinson and Company, Sparks, MD). The same procedure was carried out with the control coupons inoculated with sterile PBS, saliva, and sweat. After incubation for 24 h at 37 °C, colony-forming units were counted for each plate, averaged, and expressed as CFU/cm^2. Random colonies from spread-plated coupons were streaked on MRSA chromagar plates to confirm the lack of possible cross contamination. The culture-based enumeration was performed just after drying, and then every 24 h until no colonies were recovered from the inoculated surfaces.

Transmissibility of MRSA from Air Cabin Surfaces to Pigskin

Right after drying, two coupons from each surface/liquid were retrieved and placed aseptically into a sterile 100 x 25 mm Petri dish (VWR International, Radnor, PA). A separate pigskin swatch was pressed against each individual surface coupon for 3 sec. Each pigskin swatch was then placed into separate sterile 50 ml polystyrene conical tube (VWR International, Radnor, PA) containing 1 ml of PBS and vortexed for 30 sec. Serial dilutions of the resulting solution were prepared, and 100 µl was plated onto TSA agar plate (Becton,

Dickinson and Company, Sparks, MD) before incubation for 24 hours at 37°C. The functionality of the pigskin transmission model was assessed by inoculating 2.5 x 10^5 CFU/ml of MRSA suspended in PBS, saliva, and sweat directly onto pigskin. As a negative control, we used the resultant PBS suspension of vortexed sterile pigskin swatches pressed to surface coupons inoculated with sterile PBS, saliva, and sweat without prior inoculation with MRSA.

Statistical Analysis

Survivability and transmission data were analyzed using a general linear mixed effects model with log10 transformed CFU/ml counts as the response with a post-hoc Tukey-Kramer test. Time, surface, suspension media, and time by surface by media interactions were included as fixed effects. The SAS software for Windows (SAS Institute Inc., Cary, NC) was used to determine the statistical significance.

Results

Effect of Surface Type on the Persistence of MRSA

Our analysis indicates there was a significant difference in MRSA survival time among the different surface types studied ($p < 0.05$). The inoculated air cabin surfaces were grouped as porous and nonporous fomites. The porous fomites included the armrest, leather seat, and pocket cloth. The nonporous fomite group included the metal toilet handle, plastic tray table and plastic window shade. MRSA recovery rates across all time points were higher for porous fomites regardless the type of media used ($p < 0.05$). Among the porous fomites the

longest survival rates of MRSA were recorded for pocket cloth in PBS: seven days ($p < 0.05$). Pocket cloth was the porous surface to retain MRSA for the longest period of time in saliva and sweat also ($p < 0.05$), seven and five days respectively. Among the nonporous surfaces, MRSA survived the least amount of time on the steel toilet handle: four days in PBS ($p < 0.05$) and one day in saliva and in sweat ($p < 0.05$). The plastic tray table and window shade had no significant difference in survival time except in sweat where the window shade survived for a day longer (Fig. 6.).

Effect of Suspending Media on the Persistence of MRSA

The suspending media had a significant effect on the persistence of MRSA over time. Overall, MRSA survived the longest in PBS, and longer in saliva than in sweat (time by media interaction, PBS versus saliva $p < 0.05$; PBS versus sweat $p < 0.05$; saliva versus sweat $p < 0.05$).

Transmission of MRSA from Airplane Fomites to Pigskin

The transmissibility of MRSA was compared by surface, time, and inoculation media. The nonporous fomites plastic tray table and window shade had significantly longer transmissibility times than the rest of the studied surfaces, three and two days respectively in PBS and sweat ($p < 0.05$). Saliva had a negative effect on transmissibility from these surfaces and no bacterial counts were recovered. The porous fomites armrest, leather seat, and pocket cloth had significantly longer transmission rates in saliva but shorter rates in PBS and sweat compared to the nonporous surfaces ($p < 0.05$). To determine the functionality of

our transmission model, we inoculated 2.5 x 10^5 CFU/ml of MRSA suspended in PBS, saliva and sweat directly onto pigskin. We recovered 1.5 x 10^5 CFU/ml from PBS, 1.2 x 10^5 CFU/ml from saliva and 1.4 x 10^5 CFU/ml from sweat: 60%, 48%, and 56% of the initial inoculum for each suspending medium respectively. All negative controls inoculated with sterile PBS, saliva, and sweat and pressed with sterile pigskin showed no transmission of MRSA or pigskin resident bacteria at any time point. The percentage rates of bacterial transmission to pigskin according to their survivability on each of the airplane cabin surfaces are summarized in Table 3.

Discussion

The majority of recent research investigations of the airplane cabin have focused primarily on the presence of microbes in the air. (34-38). The potential spread of airborne life-threatening viral and bacterial pathogens within the aircraft has been documented in several reports (35-38). While infection through aerosol contamination has been reported, in the current study, we evaluated the relative risk of a community-acquired strain of MRSA MW2 as a pathogen that might survive and be transmitted in the airplane cabin *via* indirect transmission through fomites. According to the CDC and other research studies, the survival and transmission of CA-MRSA from fomites appears to play an important role in CA-MRSA outbreaks in community settings with high rates of fomite and human contact (12-14, 18, 19). Given these findings, we consider airplane travel and in particular the surfaces in the airplane cabins to represent a community setting of

considerable risk for CA-MRSA outbreaks. The role of fomites as a reservoir for the survival and transmission of CA-MRSA strains on surfaces with a variety of physicochemical properties in defined laboratory conditions are well known (13, 14). However, these conditions do not represent the environment found in the airplane, in particular, low air humidity (< 18%) and a relatively stable temperature (18-24 ºC) (39). It has been shown that higher air humidity has a negative impact on the survival of MRSA on hard surfaces in hospitals where the humidity ranges between 50 and 60% (15). Nevertheless, no data are available for MRSA persistence in drier indoor environments such as the airplane cabin. In addition, in our study we evaluate the potential of artificial human fluids to enhance the survival and transmission of the bacterium. Earlier studies reveal that MRSA persists longer on solid surfaces when drying in the presence of body fluids such as blood or pus (40). For this purpose, we used simulated saliva and sweat as suspending media and compared it to PBS.

To retrieve the bacteria from each surface coupon, we used the direct sonication method. Swab sampling is usually the method of choice because it is the most practical for field environmental sampling. However, we believe this method was inadequate for our goal, because it can suffer from a lack of sensitivity and high variation between coupon replicas since bacterial cells may become trapped within the cotton fibers of the swabs (41). In addition, recent studies have shown that sonication is a more optimal method for bacterial retrieval from biofilms on hard surfaces such as titanium and steel (42).

Our data indicate survival was more prolonged on porous compared with nonporous fomites. Among the porous fomites the longest survival rates of MRSA were recorded for pocket cloth in PBS: seven days ($p < 0.05$). Pocket cloth was also the porous surface to retain MRSA for the longest period of time independently of the inoculating media ($p < 0.05$). We speculate that porous fomites permit bacteria to colonize within the crevices of the surface which allows bacteria to locate a niche for attachment, shielded from dehydration and other stressors. The results of our study are in consensus with other reports showing longer survivability rates on porous materials such as sheets, towels, and other textile cloth materials often used in health-care facilities (13, 14, 17). On the contrary, the nonporous surfaces, such as the steel toilet handle, plastic tray table, and plastic window shade offer less protection from desiccation and oxidation. In our trials, MRSA survived the least amount of time on steel toilet handle: four days in PBS ($p < 0.05$) and one day in saliva and in sweat ($p < 0.05$). A recent investigation by Desai et al., 2011, rationalized the shorter survivability and transmission rates from stainless steel to be due to oxidation, which raises the pH of the inoculum and might have an antimicrobial effect (14). The possible presence of copper in this surface can also facilitate membrane lipid peroxidation which in turn damages bacterial membrane integrity. In addition, the steel toilet handle is also the only hydrophilic surface in our survivability and transmission study. Previous research reports have shown *S. aureus* can form biofilms more rapidly on hydrophobic than on hydrophilic substrates (43).

Our survivability trials also indicate that MRSA survival is enhanced in PBS, and is more sustained in saliva than in sweat (time by media interaction, PBS versus saliva $p < 0.05$; PBS versus sweat $p < 0.05$; saliva versus sweat $p < 0.05$) regardless of the surface used. We speculate that the presence of organic acids such lactic and acetic acid in the simulated sweat recipe that mimic the "skin acid mantle" have a negative effect on MRSA survivability. Lactic and acetic acids can penetrate through the cell wall and cytoplasmic membrane and reduce the intracellular pH, disrupting the proton motive force. The persistence of MRSA was also negatively affected when suspended in simulated saliva and compared to PBS. We hypothesize that the presence of methyl p-hydroxybenzoate (methylparaben) in the formulation has a negative effect on MRSA survivability. This chemical is widely used as antibacterial preservative in pharmaceutical, cosmetic, and food products. Parabens cause loss of the membrane semi permeability and energy uncoupling by inhibiting the uptake of metabolites (44). Survivability was longer in saliva than in sweat. We speculate that the discrepancy in survivability is due to the contrast of the pH of the two media, as well as the presence of xanthan gum in the saliva suspension. Besides as a saliva substitute this polysaccharide is often used as a microencapsulation material of probiotic bacteria for their long term preservation in harsh environmental conditions (45).

To assess transmissibility of MRSA from fomites to skin, we used the pigskin model described by Maish et al., 2007 (31). Sterile pigskin has been used in the past as a surrogate for human skin to measure for MRSA colonization (46).

However, instead of incubating the skin in 70% ethanol for 5 min, the pigskin swatches were sterilized using gamma (γ) irradiation. We believe this sterilization approach is more efficient to eliminate the resident bacteria from the skin without causing any physical alterations to the epidermal layer. Gamma irradiation has been routinely used for the sterilization of skin, bone, and other allograft tissues (32, 47).

According to our pigskin model, the nonporous fomites plastic tray table and plastic window shade had significantly longer transmissibility rates than the rest of the studied surfaces: three and two days respectively in PBS and sweat ($p < 0.05$). We rationalize that nonporous fomites have a more homogeneous surface that permits a more uniform contact with the pigskin compared to the porous fomites. In contrast, porous fomites facilitate bacteria to occupy the crevices of their uneven surface which hinders an optimal contact with the pigskin. Saliva had a negative effect on transmissibility from these surfaces and no bacterial counts were recovered. The porous fomites armrest, leather seat, and pocket cloth had significantly longer transmission rates in saliva but shorter rates in PBS and sweat compared to the nonporous surfaces ($p < 0.05$). We speculate that the presence of xanthan gum in the formulation of the simulated saliva fixed the bacteria and prevented the optimal contact with the pigskin reflected in the absence of transmissibility. Overall, our data indicates that bacterial survival was positively correlated with transmission to the pigskin over time. This supports the usefulness of our transmissibility model.

Our experimental design is not devoid of limitations. The CA-MRSA strain used in this study is not the most prevalent strain in the United States. USA300 was associated with numerous outbreaks. To reduce the risk for the laboratory personnel, we used a less common strain MRSA MW2 isolated in 1998 in a CA-MRSA outbreak in North Dakota (27). Also, in order to quantify the survivability and transmission rates we used standard plate counts. This method suffers from inadequate sensitivity and does not take into account the ability of the bacteria to survive under stress conditions in a viable but non-culturable (VBNC) state (48). As a consequence, we were limited in terms of initial bacterial inoculum, which might be higher than the actual number deposited by asymptomatic carrier onto a fomite during contact and can also affect the length of transmission. Previous studies have shown that a higher bacterial inoculum prolongs MRSA survival on fomites (49). The use of quantitative PCR and electron microscopy in a future research can contribute to a more detailed elucidation of the dynamics of MRSA survival and transmission in the airplane cabin.

In an attempt to recreate a mode of contact for transmission we pressed pigskin swatches for 3 sec to the contaminated coupons. It is unclear whether this type of contact would offer enough time and pressure for the efficient transmission to the skin. Earlier reports have indicated that friction can increase the level of *S. aureus* transfer from fomites to skin by up to five fold (50).

Conclusion

This study evaluated CA-MRSA survival and transmission from surfaces present in the airplane cabin. Our experiments indicate that this pathogen can tolerate well the dry environment in the airplane cabin, and can survive for several days on surfaces with different physicochemical properties, in the presence and absence of organic matter with a different pH. In addition, our pigskin model showed that contaminated fomites in the cabin can serve as a reservoir for transmission. To our understanding, the current study is the first attempt to elucidate the relative risk of infection for passengers during a commercial airplane flight. We believe the collected data would aid to develop more efficient and cost-effective cleaning and disinfection procedures for the airplane cabin surfaces.

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Figures and Tables

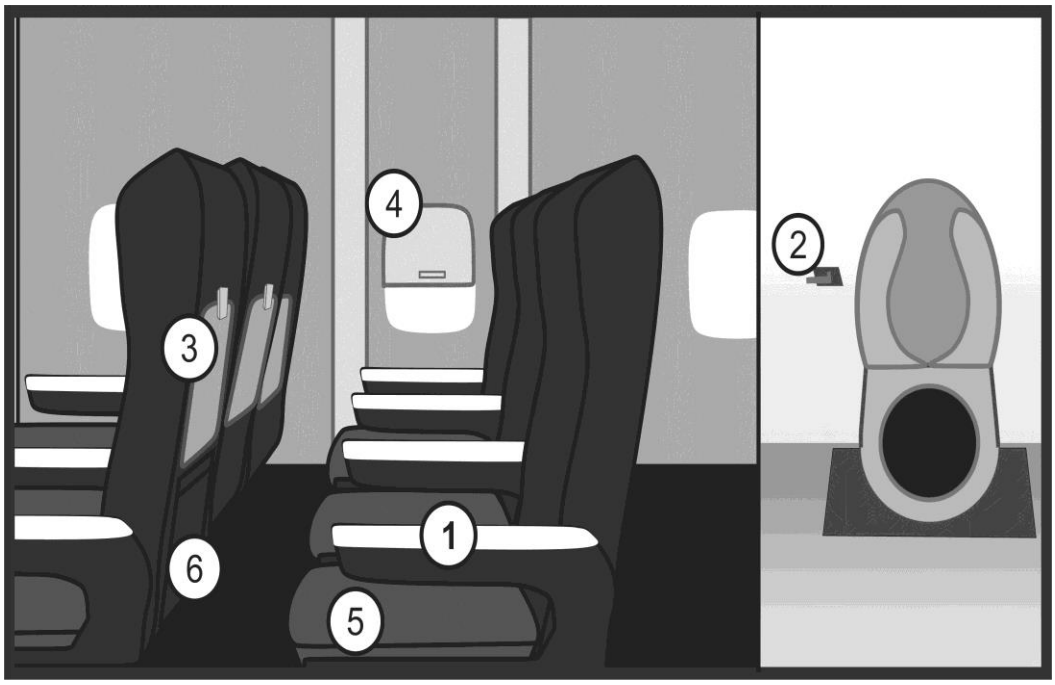

Figure 5. Airplane cabin surfaces used in this study

Rubber arm rest (1), steel toilet handle (2), plastic tray table (3), plastic window shade (4), leather seat (5), and textile pocket cloth (6).

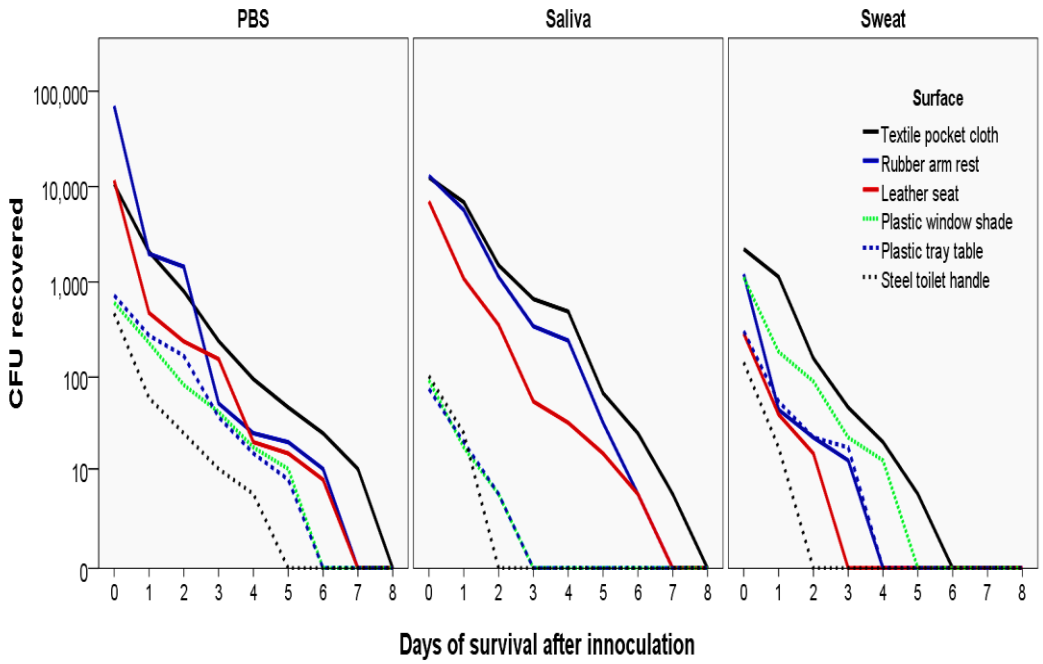

Figure 6. Survival of MRSA on six airplane cabin surfaces

The survivability counts of MRSA were obtained by plating the bacteria recovered by sonication from each surface/media every 24 h until no colonies were observed for a time point. The absolute numbers of CFU/ml were plotted against time (days).

Surface	Media	Time							
		0 h		24 h		48 h		72 h	
		Average cfu	%	Average cfu	%	Average cfu	%	Average cfu	%
Arm rest	PBS	7.9×10^2	1.1	4.5×10^2	22.8	1.3×10^2	9.2	0	0
	Sweat	3.2×10^2	26.0	2.8×10^1	61.1	0	0	0	0
	Saliva	3.1×10^2	2.4	1.3×10^2	2.2	0	0	0	0
Toilet handle	PBS	3.6×10^2	76.5	4.3×10^1	70.8	0	0	0	0
	Sweat	3.5×10^1	24.6	1.3×10^1	71.4	0	0	0	0
	Saliva	1.5×10^1	14.6	0	0	0	0	0	0
Tray table	PBS	3.5×10^2	47.6	1.1×10^2	39.1	1.1×10^2	67.2	4.0×10^1	106.7
	Sweat	2.2×10^2	69.4	4.3×10^1	77.3	1.0×10^1	44.4	0	0
	Saliva	0	0	0	0	0	0	0	0
Window shade	PBS	4.7×10^2	77.4	1.1×10^2	47.8	5.8×10^1	69.7	3.3×10^1	76.5
	Sweat	3.3×10^2	29.1	9.0×10^1	48.7	1.8×10^1	19.4	0	0
	Saliva	0	0	0	0	0	0	0	0
Leather seat	PBS	4.1×10^2	3.5	5.0×10^1	10.5	2.3×10^1	9.5	0	0
	Sweat	1.7×10^2	59.5	0	0	0	0	0	0
	Saliva	2.3×10^2	3.3	5.5×10^1	5.1	0	0	0	0
Pocket cloth	PBS	1.0×10^2	1.0	3.8×10^1	1.8	1.0×10^1	1.3	0	0
	Sweat	5.8×10^1	2.6	1.3×10^1	1.1	0	0	0	0
	Saliva	6.5×10^1	0.5	3.0×10^1	0.4	0	0	0	0

Table 3. Transmission rates of MRSA on air cabin surfaces

Transmissibility was assessed on six airplane cabin fomites in three different suspending media, measured at 0 hours (right after drying the inoculum), 24 h, 48 h, and 72 h using the pigskin model, vortexing, and standard plate counts and expressed as percentage of transmission given the survivability counts. To obtain the percentage of transfer from surface to skin the following formula was used:

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

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Chapter 4

Isolation of Phage Displayed Oligopeptides for Incorporation onto Magnetoelastic Sensor for the Detection of *Campylobacter jejuni*

Introduction

Over the past decade in the United States, clinical surveillance studies have shown bacteria, viruses and other pathogens to cause over 48 million cases of human gastro enteric infections with the related cost of $6.9 billion every year (1, 2). The five most frequent bacterial species causing disease are *Salmonella* (nontyphoidal), *Campylobacter* spp., *Echerichia coli* O157:H7, *Echerichia coli* non-O157 STEC, and *Listeria monocytogenes* (1, 3, 4). *Campylobacter* spp., in particular *Campylobacter jejuni* and its close relative *C. coli*, are the two species that are the most frequent cause of the type of human gastroenteritis known as campylobacteriosis (5). It is the most common foodborne-illness in the developed world (1, 4, 6). In the United States campylobacteriosis affects over 2.4 million people annually (1, 3). The vast majority of clinical cases are caused by *C. jejuni* 95%, followed by *C. coli* comprising 4% and other *Campylobacter* spp. accounting the remaining 1% (7).

Poultry and poultry products are considered the major cause of human campylobacteriosis (8). The birds are colonized at the age of two to three days and highnumbers of bacteria remain in their intestinal tract as commensal organisms (9). High contamination with Campylobacter, in the range of 60%, has been found in chicken meat in retail stores worldwide and more than one species can be present in the same carcass (10, 11).

Campylobacteriosis is a self-limiting disease, characterized by symptoms of severe abdominal pain, bloody diarrhea and fever (12). A serious sequel of *C. jejuni* infections is the autoimmune neurodegenerative disorder known as Guillain–Barre syndrome. This disorder affects one out of every 1000 cases of campylobacteriosis (13). Each patient with Guillain–Barre syndrome costs the United States health system $318,966, totaling $1.7 billion annually (14). The primary antibiotic for the treatment of most *Campylobacter* infections is erythromycin. Nevertheless, it has been found that 68% of *C. coli* strains are resistant to erythromycin (15). Therefore, the differentiation of *Campylobacter* species is critical in cases of severe infections requiring antibiotic treatment.

Currently, the identification of *Campylobacter* species from patient stools and chicken carcasses involves conventional culture and biochemical tests that are slow and lack sensitivity and specificity (16-18). Commercial kits based on polyclonal antibodies such as latex-agglutination require pre-enrichment and suffer from cross-reactivity (11, 19). On the other hand, the production of monoclonal antibodies is handicapped by their cost, short shelf-life and fragility when used in harsh environments (20). Although reliable, real-time multiplex

PCR based methods need expensive equipment, skilled personnel and a pre-enrichment step to reduce the inhibitory effects of the stool or food contents (21). At present, alternative rapid immunological or nucleic acid methods are only regarded as presumptive tests and must be confirmed by a standard method (16, 22). A new performance standard issued by the United States Food Safety and Inspection Service (FSIS) involves the screening of processed carcasses to limit the *Campylobacter* contaminated poultry products reaching the consumer (23).

The evident weaknesses of the current *Campylobacter* detection procedures demand the design of fast, cost-effective approaches, able to screen numerous food samples in a rapid and routine fashion to reduce the risk of campylobacteriosis. Therefore, the goal of this study was to develop a method for the rapid, sensitive and specific detection of *C. jejuni* in poultry meat. Given the resilient characteristics of filamentous phages, we hypothesize that affinity isolated oligopeptides from the pIII phage display library, coupled to magnetoelastic transducers, can be an economic and robust alternative to the current detection strategies.

Campylobacter jejuni **Overview**

C. jejuni is a Gram-negative bacterium with a spiral shape, 0.5 and 5 μm long and 0.2 and 0.8 μm wide. It exhibits a microaerophilic, capnophilic, thermophilic nature with an optimal growth temperature between 37 °C to 42 °C. The organism does not grow below 30 °C (24). *C. jejuni* does not ferment carbohydrates and obtains energy from amino acids and tricarboxylic acid (TCA) cycle intermediates (25). The organism is highly motile, possessing one or two

polar flagella contributing to the corkscrew motion essential to its virulence (26). It is negative for indole, methyl red and acetoin production, and cannot hydrolyze urea or grow in the presence of 3.5% NaCl (15). *C. jejuni* hydrolyzes hippurate. Consequently, the ability to hydrolyze hippurate can be used to distinguish *C. jejuni* from other *Campylobacter* species (27).

Genome Characteristics

In an attempt to elucidate the pathophysiology of *C. jejuni*, several strains have been completely sequenced, including NCTC11168 (1.6Mbp), RM1221 (1.8Mbp), 81-176 (1.6Mbp), and 81116 (NCTC11828, 1.6Mbp) (25, 28-31). The genomes of these strains are small, circular with a low GC content of about 30%. A common feature of these strains is the presence of homopolymeric G tracts of variable number and length in each strain (25, 28, 30, 31). These homopolymeric tracts have the potential to cause multiple phase variation events, gene duplications, deletions, frameshift and point mutations, and can contribute to the high genomic variability among different *C. jejuni* strains (25, 29). The sequence analysis revealed these homopolymeric tracts reside in genes responsible for the biosynthesis or modification of surface structures such as the capsule, lipooligosaccharides (LOS) and flagellum, and may be responsible for the immune avoidance and persistence of *C. jejuni* in its hosts, as well as its survival in the environment (25, 32-35). Besides common genomic traits, sequencing and comparative genomics revealed great genetic variability. The *C. jejuni* strain NCTC11168, a human isolate, does not contain any insertion sequences (25). In contrast, *C. jejuni* RM1221, an isolate from a market chicken carcass, does

contain insertion sequences, mainly four large genomic elements that include bacteriophage-related genes absent in *C. jejuni* NCTC11168. It was discovered that at least one of these genomic elements was present in numerous other *C. jejuni* strains of different origin which suggests temperate bacteriophages as a source of genomic diversity (29). On the other hand, strain 81-176, a human isolate, contains unique genes that confer high virulence to this strain (30). The genetic variability between strains of different origin can also be due to the fact that *C. jejuni* is naturally competent, taking DNA from other species present in the environment (25, 36-38). The organism has a nucleotide excision repair, base excision repair (BER), and recombinational repair but lacks a RecA-induced SOS response and a methyl-directed mismatch repair (MMR) system. The absence in *C. jejuni* of a RecA-induced SOS response that induces DNA repair systems, as well as the apparent absence of a MMR system, may enhance the genetic diversity and adaptive abilities of *C. jejuni* (39).

Survival of *C. jejuni* on Abattoir Surfaces and in the Chicken Meat

Contamination of meat and equipment occurs during automated slaughtering and processing as feces leak from the broiler carcasses (40). *C. jejuni* is able to remain viable on equipment surfaces after cleaning and disinfection even though the organism is sensitive to nutrient depletion, dehydration, low pH and atmospheric oxygen and lacks global stress factors such as RpoS and oxidative stress response factors such as SoxRS and OxyR (25, 41). However, *C. jejuni* does utilize enzymes such as superoxide dismutase (SOD), alkyl hydroperoxide reductase (Ahp) and catalase (KatA), which have been found to

play a major role in oxidative stress defense (42-44). In addition, the formation of biofilms on materials commonly used in industrial settings has been suggested as another mechanism by which *C. jejuni* is able to persist in the environment (45). The higher oxygen levels outside the animal host not only trigger the oxidative stress response, but also trigger the formation of biofilm which protects the bacteria from stress conditions and can serve as a reservoir of viable planktonic cells (46). Several genes have been implicated to have a role in the formation of *C. jejuni* biofilms. A knockout mutation of the *csrA* gene, a global posttranscriptional regulator, reduces the biofilm formation by 50% (47). Deletion of the *peb4* gene, coding for an adhesion protein that is up regulated during biofilm growth, obliterates biofilm formation when compared to the wild type (48). Analysis of *luxS* mutants deficient in the production of the quorum-sensing signaling molecule AI-2 has indicated that quorum-sensing (intercellular signaling) also plays an important role in the formation of *C. jejuni* biofilms (49). In addition, a proteomic analysis of *C. jejuni* 11168 biofilms showed that increased expression of proteins involved in the flagellar motility and demonstrated flagella are indispensable for biofilm formation (50). The flagella biosynthesis genes are under the control of sigma factors such as RpoD, FliA and RpoN (51-53). *C. jejuni* cannot grow on meat stored at refrigeration temperature because of its narrow temperature range for growth, the lack of microaerobic conditions and the absence of obvious cold shock proteins (24). However, *C. jejuni* remains metabolically active at 4 °C and is fully motile, retaining the capacity to move towards favorable environments (54). Immersion chilling of the

broiler carcasses, a method used by the poultry industry in the United States, helps reduce the load but does not deliver *Campylobacter* free products to the consumer, even in combination with UV treatment (55, 56). Besides biofilm formation, experimental data have implicated the chicken skin and chicken juice to have a protective effect and enhance the survivability of *C. jejuni* at refrigeration temperature (57, 58). A microarray expression analysis revealed LuxS and CfbpC, an iron-uptake ABC transporter, play key roles in adaptation and prolonged survival in the chicken meat juice environment at low temperatures (59).

When exposed to stress, *C. jejuni* undergoes morphological change from spiral to coccoid shape (60). It is not clear whether the coccoid shape is a non-viable, degenerative form or is a viable but nonculturable state (VBNC) until favorable conditions are encountered (60). Given the low infectious dose of *C. jejuni* (61) and the ability of the organism to withstand the above mentioned stressors during poultry meat production, routine monitoring, detection and identification of this pathogen is necessary to reduce its impact upon human health.

Detection Methods for *Campylobacter* species in Food

Culture Methods

The media used for the isolation of *Campylobacter* from food and water are the selective media originally designed to isolate *C. jejuni* from feces (16). Due to freezing, cooling, heating and salting of the food products, *Campylobacter* cells might be seriously injured and enter VBNC. Liquid enrichment for at least

48 hours is required to heal the cells before plating and enumerating on selective agar plates. Enrichment broths include Preston broth, Campy-thio, cold enrichment and *Campylobacter* enrichment broth (62-64). The selective agar plates incorporate cocktails of antibiotics such as cefoperazone, amphotericin B, trimethoprim and vancomycin in three types of rich basal medium: charcoal-based, blood free, and blood-based, all used at 42 °C in microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) (62). Isolation is followed by biochemical tests (16). According to the Bacteriological Analytical Manual Online of the U.S. FDA, the recovery of *Campylobacter* from foods is a labor intensive process that requires up to five days (16).

Identification of *Campylobacter* spp. using Immunoassays

The immunological methods for the detection of *Campylobacter* spp. applicable to foods or clinical or environmental samples are based on latex agglutination and enzyme-linked immunosorbent assays (ELISAs). The latex agglutination tests commercially available in United States are CAMPY (jcl) (Scimedx Corporation, Denville, NJ 07834), the Dryspot *Campylobacter* (Oxoid, Basingstoke, Hampshire, England), and the Microgen M46 *Campylobacter* (Microgen Bioproducts Ltd., Camberley, Surrey, United Kingdom). The only test approved by the Food and Drug Administration in the United States is CAMPY (jcl). The evaluation of these tests showed they are not specific enough and cannot distinguish closely related organisms and are only recommended to confirm culture results (19). Several commercial automated ELISA systems are available on the market. The system that has received the most attention is VIDAS CAM

(BioMérieux SA, F-69280 Marcy l'Etoile, France). The main problem with VIDAS CAM is the need for pre-enrichment, cross-reactivity and a detection limit similar to the one achieved with culture (11, 65). Recently, researchers have directed their efforts towards the production of monoclonal antibodies based on pursuing specific targets such as the flagellin protein FlaA (66) and one of the major outer membrane proteins (*porA* gene) of *C. jejuni* (67). The problem with using surface-exposed molecules such as the flagellar proteins, as well as the O-antigen and the capsule, is that they undergo phase variation and variable glycosylation to avoid immunological detection or as a response to an environmental stress (33, 66). Therefore, they might not be adequate as a target for detection. Furthermore, monoclonal antibodies raised through hybridoma technology have a high cost and short shelf life and are prone to degradation (20).

PCR-based Methods

Currently, most PCR detection strategies are based on real-time multiplex PCR. Real-time multiplex PCR employs fluorescent probes and primer sets targeting different gene sequences in a single reaction. Besides being highly specific, this approach allows quantification of the bacteria present in the sample tested (68, 69).However, a pre-enrichment step is often necessary to reduce the PCR inhibition by the food ingredients (21). Although the multiplex real-time PCR method is specific and sensitive, the main disadvantages are the need for trained personnel required to interpret the results and expensive equipment; thus, making the approach impractical in the economic sense.

Phage Display

Phage display is a method that takes advantage of filamentous bacteriophages such as M13, f1 and fd of the family, *Inoviridae*. They are used as cloning vectors to display foreign peptides or proteins on their surface. The outer coats of these filamentous phages are composed of 2700 α-helical subunits of the major coat protein pVIII, which encase the single stranded phage DNA. At the tips of the phage there are five copies of each of the minor coat proteins: pIII, pVI, pVII, and pIX (70) (Fig. 7). To create a phage-display library, degenerate synthetic oligonucleotides are spliced in frame into one of the phage coat protein genes. The peptides encoded by these degenerate oligonucleotides are expressed along with the major coat protein, pVIII, or the minor coat proteins, pIII or pVII, as an N-terminal fusion (70, 71). Thus, the foreign DNA insertion generates libraries with a billion different virions, each expressing a different peptide configuration (70).

Currently the most common phage display libraries are based on pVIII and pIII proteins (71). Phage libraries in which peptides are expressed on all copies of the major coat protein, pVIII, are commonly known as landscape phage libraries (71). In contrast, the pIII display strategy is based on the expression of foreign peptides on the five copies of the pIII minor coat protein at low valency (1-5 copies per virion). Besides displaying the foreign peptide fusion, the pIII protein is also responsible for the binding to the F-pilus of a recipient bacterial cell that serves as an entry receptor (72). An advantage of a landscape phage display over pIII is the multivalent display of peptides because the major coat protein, pVIII, is

present at ~2700 copies per virion of which ~10% can be reliably fused to peptides (~200 copies per virion) (Fig 7.) (71, 73). However, the multivalent display can lead to apparent high affinity due to the multiple binding events compared to the lower valency pIII display where fewer binding events can confer more specificity. Another drawback of the pVIII display is the relatively short peptide (8–9 amino acids) that can be fused to the major coat protein without affecting its function (71, 73). In contrast, pIII protein can display up to 50 peptide residues on all five copies without affecting the phage infectivity (74).

Biopanning

The procedure allowing affinity selection of phage displayed peptides that bind a given target is commonly known as biopanning (75). This technique has been used for the selection and isolation of either pVIII or pIII displayed peptides with affinity for pathogens such as *Bacillus anthracis* spores (76-78) and *Salmonella enterica* serovar Typhimurium (79, 80). Even though in these studies the displayed peptides suffered from cross-reactivity with other closely related pathogens, they highlighted the use of phage display to uncover oligopeptides that can be used as bio-recognition ligands in biosensors given the resilient nature of filamentous phage compared to the fragility of antibodies (20, 81, 82). The employment of landscape phage display in conjunction with magnetoelastic sensors for the above mentioned pathogens has been tested and optimized (83, 84). In more recent studies, magnetoelastic sensors covered by landscape phage displayed peptides and immobilized by simple physical absorption were tested with spiked water, non-fat milk and tomatoes (73, 76, 83). Despite the

interferences created by the food matrix, these studies proved very sensitive and were able to detect their spore and bacterial targets with concentrations as low as $1x10^3$ CFU/ml. Magnetoelastic transducers are made of ferromagnetic alloys of nickel, molybdenum and boron (85). When a magnetic field is applied, the sensors oscillate mechanically at a specific base frequency. When an analyte binds to the sensor, it results in a frequency change that can be measured and quantified. These magnetoelastic strips have a low cost of production, can be operated wirelessly and can give a real-time measurement of the binding events (86). Given the difficulties of the current methods of *Campylobacter* spp. detection, this project's intent is to offer an economic alternative for the detection and screening of these pathogens in foods by integrating phage display technology and magnetoelastic transducers.

Materials and Methods

Phage Library

The current study takes advantage of the commercially available phage display library Ph.D.12 (New England Biolabs, Ipswich, MA). This library is derived from the cloning vector M13mp19, which carries the *lacZ* gene. The library contains approximately 10^9 independently displayed peptides, only a fraction of the 4.1×10^{15} possible 12-mer sequences (87). It consists of N-terminal fusions of 12 random amino acids to the five copies of the minor coat protein pIII of each phage clone. This is achieved as a result of insertion of a random 36-base DNA fragment into a cloning site at the start of the gene encoding the pIII protein of the phage (88). Therefore, the sequence of any

displayed peptide that binds a desired target can be determined by sequencing of the peptide-encoding region of the phage genome. In this study, the amino acid sequences of phage candidates with affinity for *C. jejuni* were obtained by amplification of their single-stranded genomes using -96pIII primer, provided by New England Biolabs and isolating the DNA sequences with the Spin M13 Kit (Qiagen, Valencia, CA). After automated DNA sequencing the peptides were deduced using Chromas Lite 2.01.

Phage Titering, Growth and Purification

In this study, when titering or propagation of the library was needed, the host used was *E. coli* ER2738. This strain is an F^+, *recA*$^+$ strain and the F-factor contains a mini-transposon which confers a tetracycline resistance. To obtain a biological titer, the host ER2738 is grown until mid-log phase (OD600 ~ 0.5) and infected with different dilutions of the phage library. After the infection, the host is spread on LB-IPTG plates. Since the recombinant phages from this library carry the *lacZ* gene, only the cells infected form blue colonies when plated on LB-IPTG plates (62). The amplification of the library follows the infection procedure described. Instead of plating, the infected host is grown in a shaker-incubator for 4.5 h at 37 °C. The supernatants containing the phage library are recovered by centrifugation at 14000 rpm for 10 min and the supernatants containing the library are purified by two consecutive overnight precipitations with polyethylene glycol (PEG), at 4 °C. To isolate individual clones, infection is done with individual blue colonies picked from a whole library spread plate, followed by the growth and purification steps described. The propagation and tittering steps followed the

procedures outlined in the Ph.D.™ Phage Display Libraries-Instruction manual (New England Biolabs, Ipswich, MA).

C. jejuni Strains

The biopanning experiments in this research used nine *C. jejuni* strains isolated from chicken meat from retail grocery stores in Auburn, AL (89) and provided by Dr. Omar Oyarzabal (Institute for Environmental Health, Inc., Lake Forest Park, WA 98155). All strains were previously identified using multiplex PCR assays and subtyped using a pulsed-field gel electrophoresis (PFGE) protocol (89, 90). These *C. jejuni* strains were chosen as relevant for the isolation of specific oligopeptides for the detection of *C. jejuni* in chicken meat.

Phage Library Depletion with Plastic and Bovine Serum Albumin

In this study, the depletion of plastic and bovine serum albumin (BSA) binders was achieved by the following steps. An aliquot of the primary Ph.D.12 phage library was incubated in four empty wells (100 µl per well, $1x10^{11}$ vir/ml in TBS, 50 mM Tris–HCl (pH 7.5), 0.15 M NaCl) of a 96-well polystyrene ELISA (Costar 9018) flat bottom plate (Corning, Inc., NY, 14831) for 1h at room temperature with gentle rocking. Afterwards, the phage solution from each well was transferred to a new set of four wells on the same plate. This step was repeated five times. The BSA (Sigma-Aldrich, MO 63178) depletion was achieved by blocking a new set of four wells of the same ELISA plate with 100 µl of 5mg/ml BSA in TBST (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% Tween-20 for 1h. The BSA was discarded and the wells were washed five times

with TBST. The plastic depleted library was then transferred to the BSA blocked wells and incubated for 1h. As for the plastic depletion, this step was also repeated five times, each time transferring the library to a new set of four wells blocked with BSA. After the plastic and BSA depletion steps a portion of the library was saved for titering to determine the yield of phage clones after these steps.

Affinity selection of phage displayed peptides

As mentioned earlier, the procedure allowing affinity selection of phage displayed peptides that bind a given target is commonly known as biopanning. It consists of immobilization of the target of interest on a platform and its consequent exposure to an aliquot of the phage library. After several rounds of biopanning, peptides that bind to the desired target with the highest affinity and specificity are subsequently identified by sequencing of the DNA inserts of the selected clones. If the experiment has been successful, one or more consensus sequences will be identified even without previous knowledge of the structure aimed. In this study, since none of the *C. jejuni* isolates grew well in liquid broth, before each biopanning experiment each strain was recovered from a glycerol stock (Brucella broth with 20% glycerol and 5% lysed horse blood) on several Brucella agar plates containing 5% lysed horse blood. The plates were placed in a microaerobic jar (BBL GasPak™ Systems) with a CampyGen pouch (Remel Inc., KS 66215). The jar was incubated at 42 °C, for 48 to 72h. The colonies of each strain were scraped out from the plates, resuspended in 10 ml of phosphate buffered saline (PBS), (0.15 M NaCl, 5 mM NaH_2PO_4) and centrifuged for 10

min at 6000 rpm (Eppendorf AG, rotor F34-6-38, Hauppauge, NY 11788) for three times, each time replacing the supernatant with new PBS. Each strain concentration was adjusted ($1x10^9$ cells/ml) using disposable hemocytometers (C-chip DHC-N01-5, INCYTO Co, Ltd.). A cocktail of all *C. jejuni* strains was prepared by mixing equal volumes of each strain (1 ml) in a 50 ml centrifuge tube. An aliquot of 1ml *C. jejuni* cocktail suspension was spread onto an empty sterile 35 mm Petri dish (Beckton Dickinson and Company, Franklin Lakes, NJ, 07417-1886) and dried overnight at 37 °C. On the next morning the dish was blocked with 5mg/ml BSA in TBS, for 1h at room temperature and washed 5 times with 1ml TBST. A 400 µl aliquot of previously plastic and BSA depleted Ph.D.12 library was added to the dish and incubated for 1h at room temperature with gentle rocking. Afterwards, the phage solution was removed and the dish was washed 5 times with 1ml of TBST. Phages bound to the *C. jejuni* cocktail were eluted with 400 µl of elution buffer (0.2 mM glycine-HCl, 1 mg/ml bovine serum albumin, pH 2.2) and added to the Petri dish for 10 min. The eluate was transferred to a microcentrifuge tube and neutralized with 75 µl 1 M Tris–HCl (pH 9.1). The second and third biopanning were done following the same steps. After each biopanning, a portion of the library was saved for titering to determine the titer yield of clones binding to *C. jejuni*. The current protocol of biopanning is a modified procedure already described by Sorokulova, *et al*., 2006.

Library Depletion with *C. coli, C. lari* and other Foodborne Pathogens

After the first biopanning, the Ph.D.12 library was depleted with *C. coli* (ATCC 43134) and with *C. lari* (ATCC 35221). Prior to this step the library was

amplified. The growth and concentration adjustment of these two strains ($1x10^9$ cells/ml), followed the same procedure used for the growth of the *C. jejuni* strains. Instead of using cocktail mixtures, these two species were deposited on two 35 mm Petri plates per separate. After drying the cells on the plates overnight, both dishes were washed five times with TBST and blocked with 5mg/ml BSA in TBS for 1h at room temperature. The BSA was removed and the Petri plate washed five times with 1ml TBST. A 1ml aliquot of the first round biopanning amplified phage library ($1x10^{11}$ vir/ml) was added to each plate consecutively and incubated with each *C. coli* and *C. lari* for 1h, respectively. The unbound phage was collected from each plate and transferred to a microcentrifuge tube for titering. Elution buffer was not used, since the phages that bound during this depletion step were potentially cross-reacting with *C. coli* and *C. lari.* After the second biopanning the library was depleted with two non-related foodborne pathogens such as *S.* Typhimurium (ATCC 13311) and *E. coli* O157:H7 (ATCC 43894). Both were grown overnight in a shaker-incubator (18h, 200 rpm, 37 °C) in LB broth. After adjusting their concentration by centrifugation ($1x10^9$ cells/ml), the cells were dried overnight on two separate 35 mm Petri plates, at 37 °C. Plates were washed with TBST and blocked with 5 mg/ml BSA in TBS. A 1ml aliquot of the second round biopanning amplified phage library was added to each plate and incubated separately with each *S.* Typhimurium and *E. coli* O157:H7, respectively. The unbound phages were collected from each plate and transferred to a microcentrifuge tube for titering.

Results and Discussion

As discussed earlier, the Ph.D.12 phage display library contains approximately 10^9 independently displayed peptides. Usually, the vast majority of these peptides are target-unrelated sequences. In order to ensure the isolation of clones with higher specificity for *C. jejuni*, an initial depletion of the library before the rounds of biopanning was necessary. In this study, preliminary depletion of the Ph.D.12 library was done with plastic and with BSA. This preliminary depletion is essential since other studies have reported the isolation of clones binding to those entities (91, 92) and the presence of such binders can lead to false positive results with the ELISA procedure. On the other hand, the value of plastic depletion is somewhat handicapped by the natural tendency of proteins to absorb to polystyrene surfaces via hydrogen bonds and nonpolar interactions. Plastic binders are characterized by the frequent presence of aromatic amino acid residues within the displayed peptide sequence (91). Therefore sequencing of a subpopulation of clones between each round of selection can verify their presence. In the context of developing a biosensor based on phage display with low cross reactivity, more depletion rounds with plastic, BSA and a more ample list of foodborne pathogens were considered. However, a conservative strategy was chosen since extensive depletion can lower the phage library's phage titer and the overall diversity of the displayed peptides. The decrease in titer requires amplification of the library. Amplification of the library was avoided because of possible selection and amplification of fast growers or wild type phage, rather than specific target binders (93). As summarized in table 4, the depletion with

plastic and BSA reduced the titer of the library by 50%. The first round of biopanning reduced even more massively the phage titer from the initial input from $1x10^{11}$ to $1x10^{7}$ vir/ml, only 0.01% of the initial population was eluted after this step. Assuming each clone was represented equally (10^{9} different peptide combinations with initial input of 10^{11} vir/ml) there were approximately 100 phage particles per peptide combination. At this point very few clones with affinity for *C. jejuni* remained in the library, and in order to enrich this subpopulation, the library was amplified and the yield was $3\ x10^{11}$ vir/ml. After the first biopanning, to reduce the isolation of clones binding to other closely related *Campylobacter* spp., the Ph.D.12 library was depleted with *C. coli* and with *C. lari.* This depletion step was included because *Campylobacter* spp. other than *C. jejuni* are often found in the same chicken carcass and it is important to distinguish different species to assign proper treatment in case of campylobacteriosis (15). The input for this depletion step was $3x10^{11}$vir/ml; output $5x10^{9}$ vir/ml, immediately followed by the second round of biopanning with recovery $1x10^{8}$ vir/ml. The library was amplified one more time and depleted with the *S.* Typhimurium and *E. coli* O157:H7. This step was included in this late stage in an effort to reduce cross reactivity with other pathogens that are also found in chicken meat products (94). The recovered library went through a final third round of biopanning without amplification. A fourth round of biopanning was not completed because some colonies grew colorless, which indicated the selection process was moving towards isolation of wild type phage.

In this study, a phage ELISA procedure was used to evaluate the depleting steps and biopanning rounds towards a subpopulation of clones with higher affinities for *C. jejuni*. The performance of these clones was compared to the negative control M13KE vector phage, containing the *lacZ* gene but no insertion in the N-terminal sequence of pIII. A 96 well ELISA plate (Costar 9018) was used to immobilize a *C. jejuni* cocktail solution (100 μl of 1×10^9 cells/ml per well). The cells were dried at 37 °C overnight and the next morning the plate was blocked for 1h with 5mg/ml BSA in TBS (100 μl). The wells were washed five times with 100 μl of TBST. The phage eluates of the three rounds of biopanning ($1x10^{11}$ vir/ml in 55 μl TBS) were added in separate wells and the plate was incubated for 1h with gentle rocking. The phage solution was removed and each well was washed five times with 100 μl TBST. An anti-M13 rabbit polyclonal antibody (ab6188, Abcam, Cambridge, MA) was pipetted and incubated for 1h to react with *Campylobacter* phage complexes (45 μl/per well). The plate was washed again five times with 100 μl of TBST and an anti-rabbit antibody (ab6790, Abcam, Cambridge, MA) conjugated with alkaline phosphatase (AP), (45 μl/per well) was added to each well and incubated for 1h. Afterwards, each well was filled with 90 μl of NPP (p-nitrophenyl phosphate) substrate and read on a kinetic plate reader (iMark™ Microplate Absorbance Reader 168-1130, Bio-Rad Laboratories, CA 94547) at 450nm for one hour. Each round of biopanning was tested in duplicate in two separate experiments (Fig. 8).

As shown in (Fig. 9), the ELISA signal increased significantly with each round of biopanning and the selection was favored toward phages with higher affinity for *C. jejuni* compared to the negative control M13KE phage. The binding of *C. jejuni* was higher compared to the binding of the library with *C. coli* and *C. lari*. The depletion step with *C. coli* and *C. lari* after the first biopanning did contribute to a significant gain of specificity, but the cross-reactivity with *C. coli* actually increased over the course of the selection. The analysis of individual clones using the ELISA procedure can be more indicative of the successes of the pre-depletion process because certain clones with non-specific binding affinities can influence the signal intensities obtained with other *Campylobacters*.

The described ELISA procedure was also used to assess the binding affinities of individual clones. The phage library eluate of the third and final round of biopanning was isolated on an LB-IPTG plate and individual blue colonies were transferred via the pick and patch technique on a separate gridded and numbered plate. After the growth and purification, each phage was titered to determine the concentration. A total of twenty-four clones were tested for their binding affinity with *C. jejuni* cells and cross- reactivity with plastic and BSA was compared to the negative control of M13KE vector phage. Wells incubated with *C. jejuni* cells incubated without phage clones but with the primary and secondary antibody conjugated with AP were used as a positive control (Fig. 10.). This group of clones was sequenced and the amino acid arrangement of each clone was obtained using Chromas Lite 2.0.

The design of the biopanning experiments and the evaluation of the clones with specificity for *C. jejuni* with the ELISA assays, were based on exposing the phage library or individual clones to bacteria immobilized by drying. Using this method the probability of isolating peptide sequences that are specific for a particular surface structure is reduced, since this process exposes not only surface but also intracellular epitopes of the bacterium. In addition, the current experimental design increases the likelihood of isolating self-precipitating or aggregating phages, rather than peptides binding to the immobilized cells. Isolation of clones with such characteristics is difficult to avoid and the selected binders have to be tested with their target in solution. In the current study only the clones with obvious reduced cross-reactivity with plastic, BSA, *C. coli*, *C. lari*, *S.* Typhimurium and *E. coli* O157:H7 would be considered for further analysis. The sequence analysis of the subpopulation of clones is expected to show common amino acid motifs conserved after the third round of biopanning with an absence of clones containing excessive numbers of aromatic residues indicative of plastic binders. A lack of common amino acid sequences would suggest that the selection process has not applied enough pressure towards selecting clones with higher binding affinities or the selection process was too stringent allowing a competitive advantage of fast growing clones.

The ELISA analysis revealed certain clones with high binding affinities for *C. jejuni*, as well as clones with high cross reactivity with plastic and BSA (light grey bars) (Fig. 10.). The sequencing analysis of all twenty four clones showed few amino acid combinations, like serine and proline, and serine and

lysine, being predominant among different clones (Table 5.), indicating the selection process has exerted pressure towards certain combinations that remained prevalent after the last biopanning. Repeating peptide combinations were labeled with different color. The sequences lacked excess of aromatic residues commonly found among plastic binders. All clones and their respective peptide insert exhibited basic charge, except clone six with a negative charge. None of the clones was repeated among the clones sequenced. A larger population of sequenced clones is required to expose the saturation of the library with peptides with conserved motifs after the rounds of biopanning. If the sequence analysis of more clones does not achieve the expected motifs between rounds of biopanning, an extra round of biopanning should be performed. In the current study, a fourth round of biopanning was avoided because of the appearance of colorless colonies, indicative of wild type phage. It is possible that the lack of common motif in the current subpopulation represents the isolation of binders attaching to *C. jejuni* with low specificity as a consequence of the massive pre-depletion and depletion steps before and between biopanning rounds. A total of six clones (clones 6, 7, 12, 17, 21 and 24) with significantly lower cross-reactivity with plastic and BSA (black bars) were chosen for further analysis with other selected bacteria (Fig. 11). These clones however did not exhibit common motifs with the rest of the population sequenced. It is possible their overall charge contributes to the binding. The ELISA assays with these clones showed very low cross reactivity with *E. coli* O157:H7 and *S.* Typhimurium, but high cross-reactivity with *C. jejuni* and *C. coli*. Clone 17 (black bars) showed the least amount of cross

reactivity and was chosen for further analysis with competitive ELISA and co-precipitation assays.

The competitive ELISA assay is based on the ELISA assay with the following modifications. The phage clone 17 was introduced to each well containing dried *C. jejuni* in the presence of TBS aliquots containing 10, 50 and 90% of *C. coli* or *C. lari* and the results were compared to the negative control of M13KE. The goal of this test was to determine the selectivity of clone 17 in the presence of competitive ligands. In addition, a co-precipitation assay was performed (Fig. 12). In co-precipitation, 200μl of *C. jejuni* cells ($1x10^9$ cells/ml in TBS) were mixed with 200 μl of the best binding phage, clone 17 ($1x10^{11}$ vir/ml), in a microcentrifuge tube and left to co-precipitate on a rotator for 1h (three replicas). Afterwards, the *C. jejuni* phage mixture was centrifuged at 14,000 rpm for 10 min. The supernatant was discarded and the pellet was washed three times with 200 μl TBST. An aliquot of 200 μl of elution buffer was added to the microcentrifuge tube to break the *C. jejuni* phage complexes and incubated for 10min on a rotator. The tube was centrifuged at 14,000 rpm for 10 min to precipitate the cells. The supernatant, containing released clone 17, was transferred to a new microcentrifuge tube and neutralized with 38 μl Tris-HCl (pH9.8). The output of the phage titer was determined and the concentration was expressed as a percentage average of three replicates (input/output vir/ml x 100).

The major goal of the competitive ELISA and co-precipitation assays is to determine the selectivity of phage clone 17 for *C. jejuni* cells in the presence of an

increasing concentration of competitive ligands such as *C. coli* and *C. lari*. The expected outcome is a decreased cross-reactivity with *Campylobacters* other than *C. jejuni*. However, the experiment confirmed cross-reactivity with other *Campylobacters* when mixed with them in solution. Since the biopanning and depletion experiments targeted dried cells, the introduction of non-lysed cells presents different surface epitopes that might have interfered with the binding to *C. jejuni*. This problem can be observed also with the co-precipitation assay with even higher magnitude. Surface structures like the capsule and the lipooligosacharide that cover and protect the cells can have high similarity between different *Campylobacter* species and can interfere with the binding affinity of clone 17. Another drawback of the co-precipitation assay is the variation between replicates within the experiment due to the weaker affinity interaction of clone 17 when binding in liquid, and the washing procedure with cells that were not immobilized, preventing consistent input/output recovery ratios.

The competitive ELISA assay proved to have higher binding of clone 17 to *C. jejuni* cells compared to the control phage, but also confirmed the cross-reactivity with *C. coli* and *C. lari* (Fig. 12) observed in the previous ELISA experiments. The 10% aliquot mixture with both closely related *Campylobacte*r spp. did not interfere considerably in terms of binding compared to the previous assays with *C. jejuni* cells only. However, significantly lower binding signals were obtained from the 50% and 90% mixes, indicating this phage clone was interacting with both *C. coli* and *C. lari* in solution and some of the phage was

lost during the washing procedure as phage-bacteria complexes. Besides cross-reactivity with other *Campylobacters*, the co-precipitation assay revealed little correlation with the ELISA data (Fig. 13). There was no significant difference in binding with *C. jejuni*, *C. coli* and *C. lari* and no significant difference of binding with the M13KE phage. The high variance between replicas in the co-precipitation assay was probably due to the inefficient binding of clone 17 to the presented target when tested in solution. In addition, the design of the experiment requires washing procedures during which a large portion of the phage-bacteria complexes are lost.

Conclusion

The phage clones isolated in the current study from the commercial Ph.D.12 display library show potential to be used as ligands for detection and separation of *Campylobacters* from foods. The depletion and biopanning steps used throughout the experimental work proved to be important for the isolation of a subpopulation of clones with higher specificity for *Campylobacters* and low cross-reactivity with other enteric bacteria. In this study, three rounds of biopanning were completed. The sequencing analysis showed amino acid combinations, like serine and proline, and serine and lysine, being conserved among different clones (table 2). Prolines have been shown to be important for protein-protein through hydrophobic interactions (95). In addition, a larger pool of clones should be sequenced after each round of biopanning in order to confirm the frequencies of these peptides and their prevalence after each step of the selection

process. A fourth round of biopanning can exert more selective pressure and can also contribute to further enrichment with more valid clones for the specific detection of *C. jejuni*. The current experimental work failed to deliver a phage clone specific for *C. jejuni*. The isolated clone 17 with displayed peptide QGAQARSGTPVQ showed a high degree of binding with two other members of the genus *Campylobacter*: *C. coli* and *C. lari.* The lack of specificity of the isolated clone is probably due to the high similarity of surface polysaccharide structures of the capsule, the lipooligosaccharide (LOS) or other conserved epitopes, such as the outer membrane proteins. The outer membrane protein porA has been shown to be present in *C. jejuni, C. coli*, *C. lari, C. hyointestinalis* and *C. upsaliensis,* and the cholera toxin raised antibody cross-reacts with these species (96). A heat treatment to remove the capsule of the *Campylobacter* cells prior to the ELISA and the co-precipitation assay experiments can be used to confirm whether its absence can reduce the observed cross-reactivity of clone 17. In addition, an SDS-PAGE of *C. jejuni* and *C. coli* whole cell lysates and Western blot would allow identification of the protein epitope to which the phage particle binds. Given the specificity problems observed, the isolated clone 17 can be used as a test for the detection of the *Campylobacter* genus. Therefore, clone 17 should be tested for cross-reactivity with other species within the *Campylobacteraceae* family, such as *A. cryaerophylus, A. butzleri* and *A. skirrowii*, often isolated from chicken carcasses (15). A lack of cross-reactivity with these members of the *Arcobacter* genus would allow the use of this clone as a preliminary test for *Campylobacters* in foods in combination with a sensitive detection platform using

a magnetoelastic particle. The specificity to the species level, or confirmatory test can be achieved using a PCR assay.

Figures and Tables

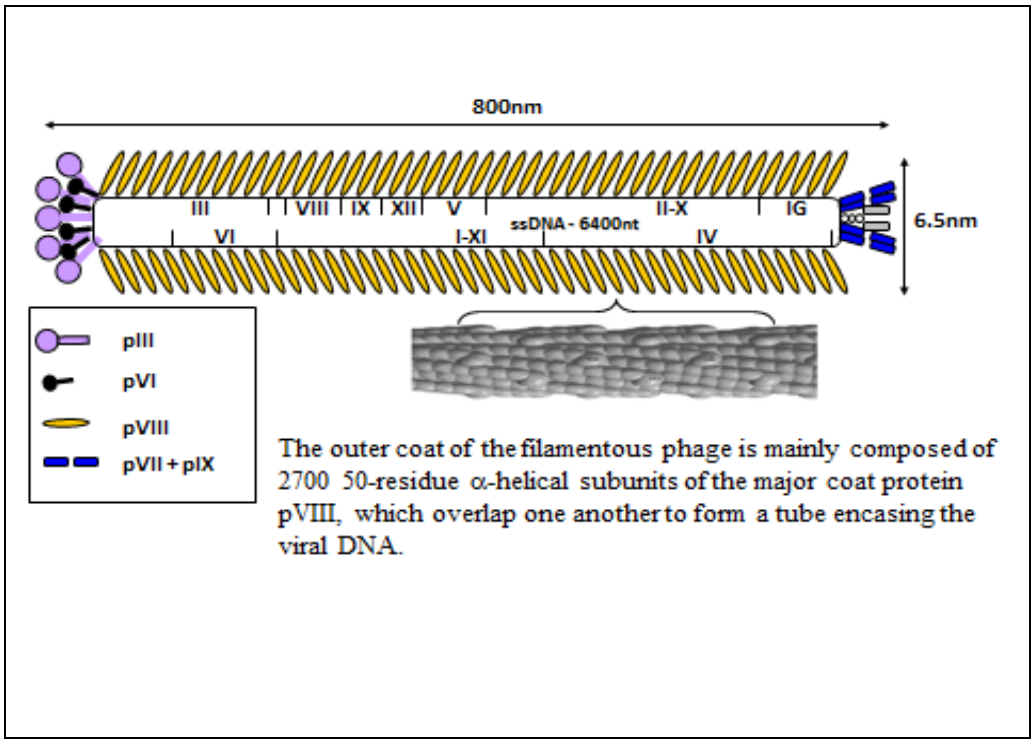

Figure 7. Structure of filamentous phages M13, f1, fd thread-shaped bacterial viruses (Smith and Petrenko, 1997).

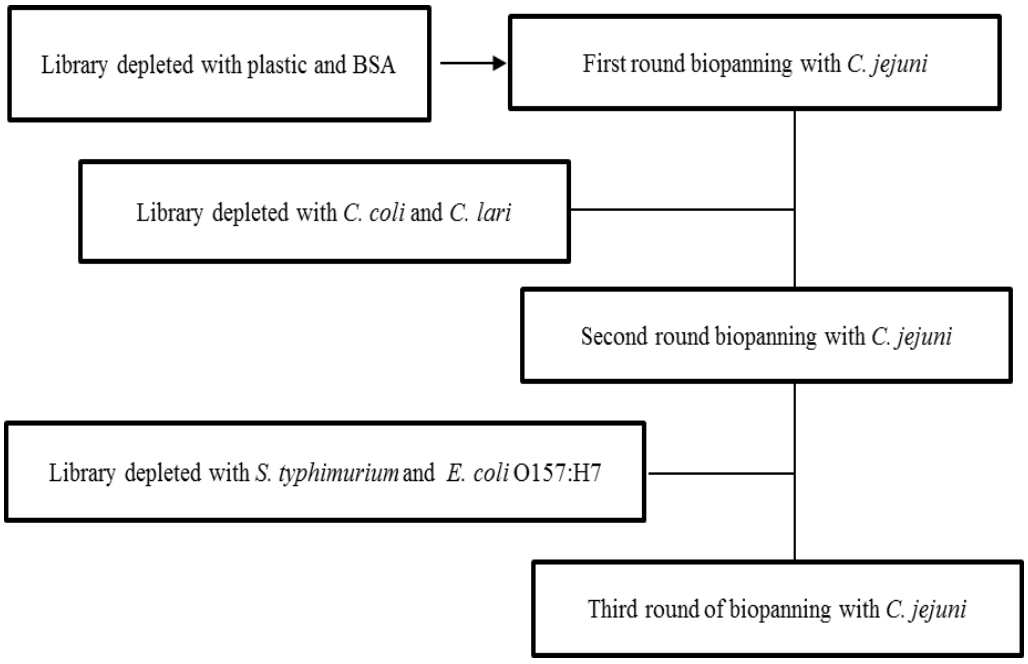

Figure 8. Depletion and biopanning procedures followed

Procedure	Depletion plastic /BSA	1st Biopanning	Library amplification	Depletion *C. coli* /*C. lari*	2d Biopanning	Library amplification	Depletion *S.* Typhimurium and *E. coli* O157:H7	3d Biopanning
Input vir/ml	$1x10^{11}$	$5x10^{10}$	$1x10^{7}$	$3x10^{11}$	$5x10^{9}$	$1x10^{8}$	$1x10^{11}$	$1x10^{10}$
Output vir/ml	$5x10^{10}$	$1x10^{7}$	$3x10^{11}$	$5x10^{9}$	$1x10^{8}$	$1x10^{11}$	$1x10^{10}$	$1x10^{9}$

Table 4. Recovery of phages after depletion and biopanning selection

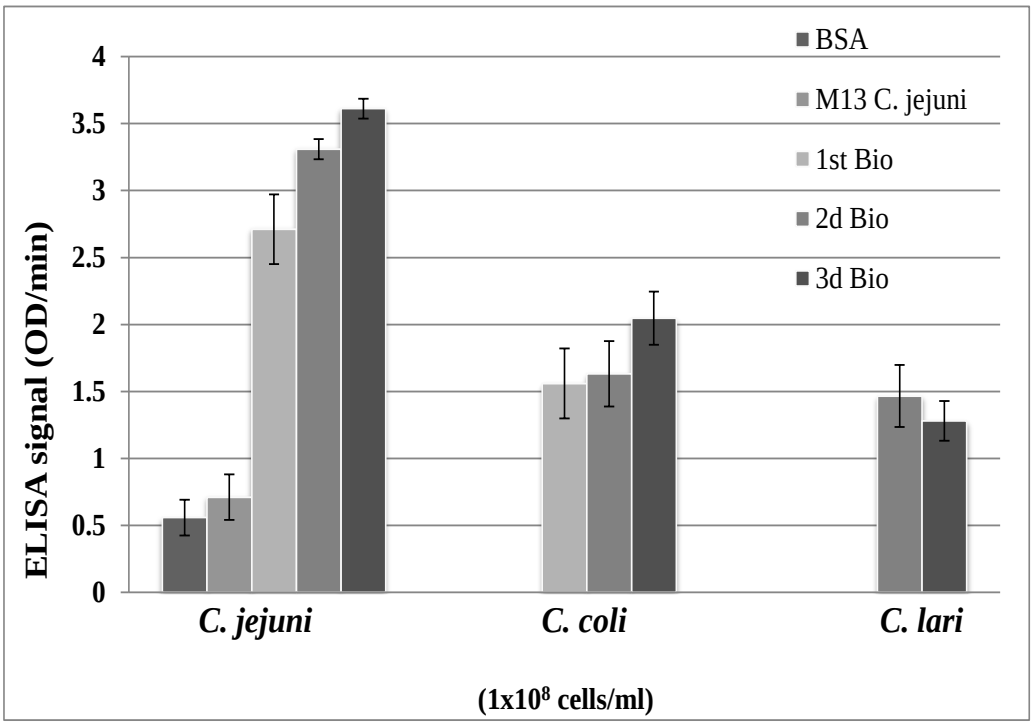

Figure 8. ELISA assessment of the pre-depletion and biopanning rounds

An ELISA procedure was performed to asses the selectivity of each step of the biopanning procedure towards selection of phages that bind preferably *Campylobacter jejuni,* rather than BSA, *Campylobacter coli* and *Campylobacter lari*. The bars represent standard deviations of two separate experiments with three replicas.

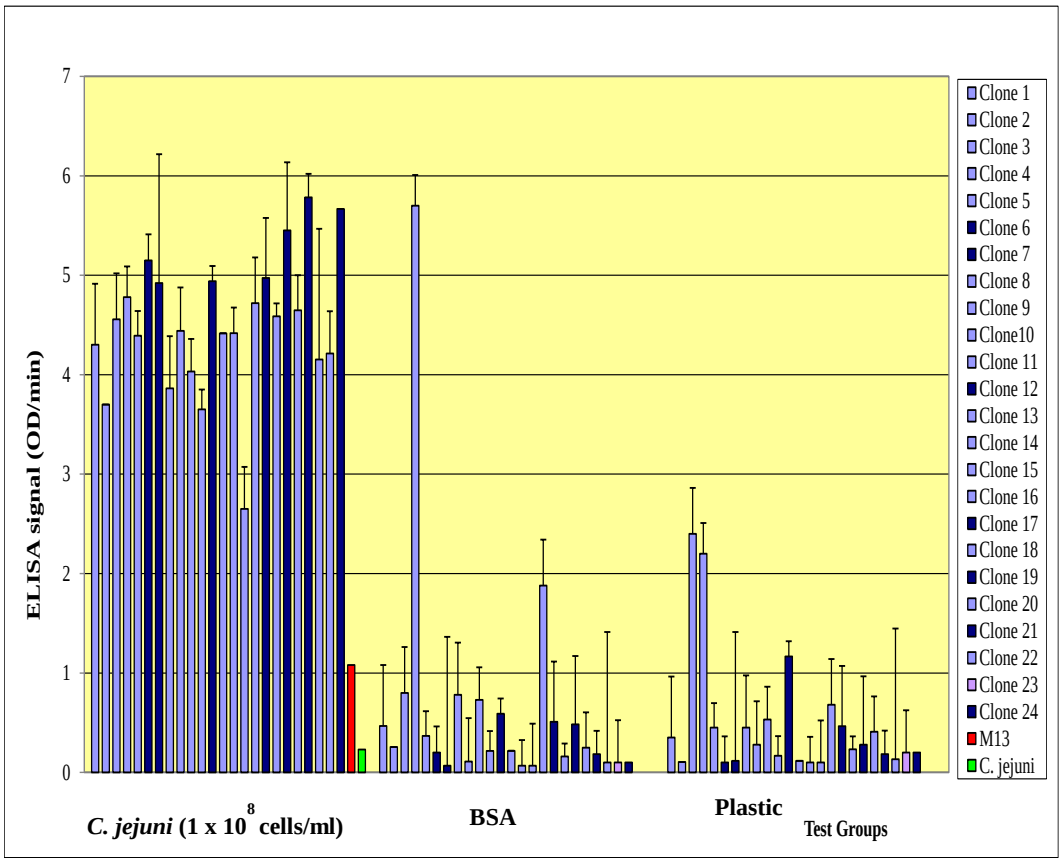

Figure 9. ELISA of individual clones following the 3d round of biopanning

Twenty-four random phage clones were selected to test the specificity and affinity to *C. jejuni* cells following the 3d round of biopanning. The red and green bars represent ELISA signal from the vector phage M13 and the signal from the antibodies incubated with *C. jejuni* cells respectively. The light blue bars represent signals from the phage clones after the 3d round biopanning; the dark blue bars indicate phages with a higher ELISA signal and low reactivity with two control groups, BSA and plastic.

Clone	Peptide sequence	Clone	Peptide sequence
1	HS**YSTSLITLYPFN**GGG	13	HS**TLQSRIVPPVEP**GGG
2	HS**SPPHVPLGWADR**GGG	14	HS**WTKNSSPTGYPR**GGG
3	HS**NGKVPDAALPRF**GGG	15	HS**SHVNIKQSPQLL**GGG
4	HS**VSPPKMTTPPTP**GGG	16	HS**TYHKPQSVPHGY**GGG
5	HS**NTTPKSPHSPEFG**GG	**17**	HS**QGAQARSGTPVQ**GGG
6	HS**IEWSDNNNFDPR**GGG	18	HS**NLYNQHEPLLAP**GGG
7	HS**DLNHSQIITSLE**GGG	**19**	HS**AQSHNQHNFNTT**GGG
8	HS**SLSPETDTPSAL**GGG	20	HS**FSPKITYPVHAS**GGG
9	HS**NIGIHTAPFSPS**GGG	**21**	HS**SVNTSYSTLNSL**GGG
10	HS**SLVTYMDYRLPA**GGG	22	HS**NLTGLPIPHNLV**GGG
11	HS**SHVNIKQSPQLL**GGG	23	HS**LTTRCSPEAQCT**GGG
12	HS**TSNLKWTPLART**GGG	24	HS**GSSFAPRDILGT**GGG

Table 5. Amino acid sequences of 24 phage clones isolated after the 3rd round of biopanning

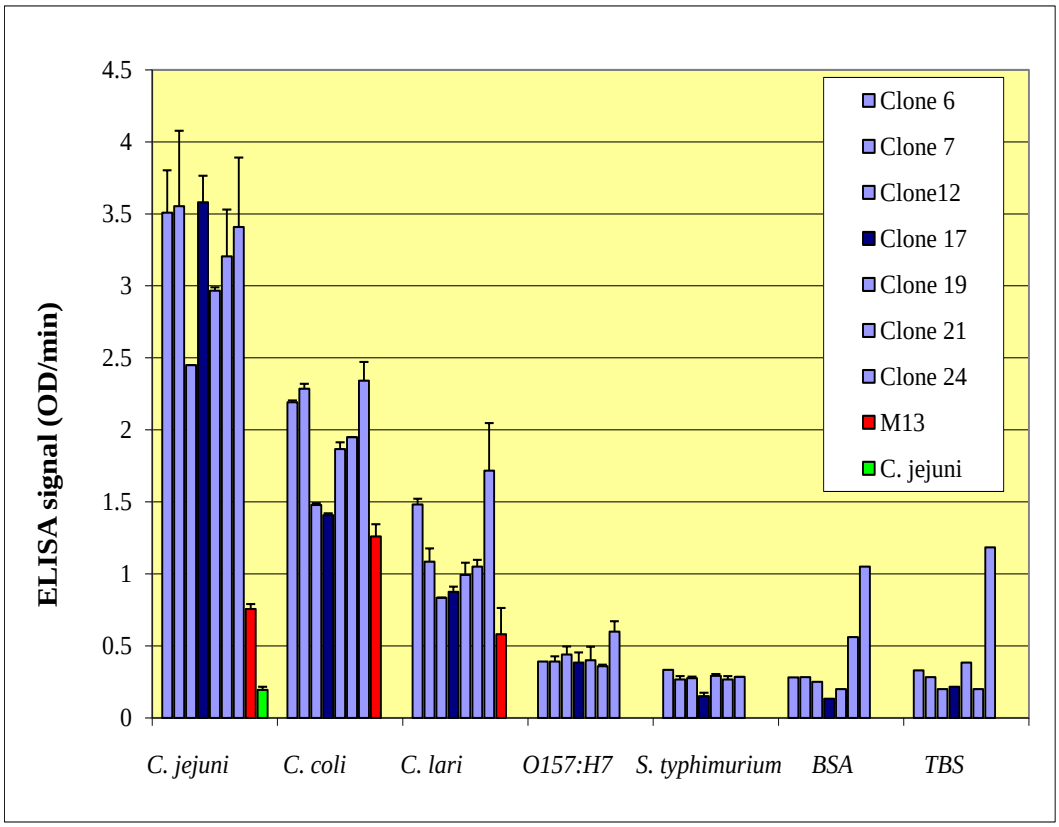

Figure 10. ELISA assessment of the specificity of seven selected phage clones

Affinity for *C. jejuni* of the seven "best" binding clones was monitored by ELISA. Cross-reactivity was tested with the closely related *C. coli* and *C. lari*, as well as *E. coli* O157:H7 and *S.* Typhimurium. The binding was compared to the binding of M13 vector phage. The clones were also tested for binding to plastic and BSA. Phage clone 17 showed highest affinity for *C. jejuni* and lowest cross-reactivity.

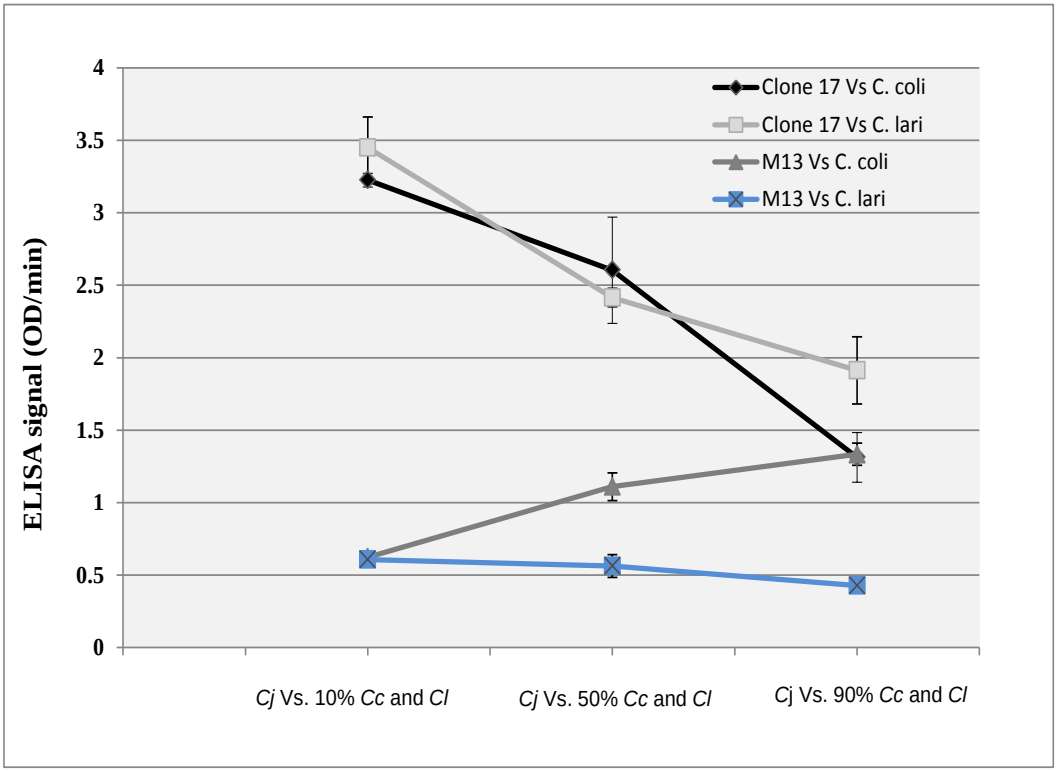

Figure 11. Competitive ELISA

The relative specificity of phage clone 17 for *C. jejuni* (*Cj*) was tested via competitive ELISA using *C. coli* (*Cc*) and *C. lari (Cl)* as competitive ligands at 10%, 50% and 90% concentrations. M13 vector phage served as a negative control.

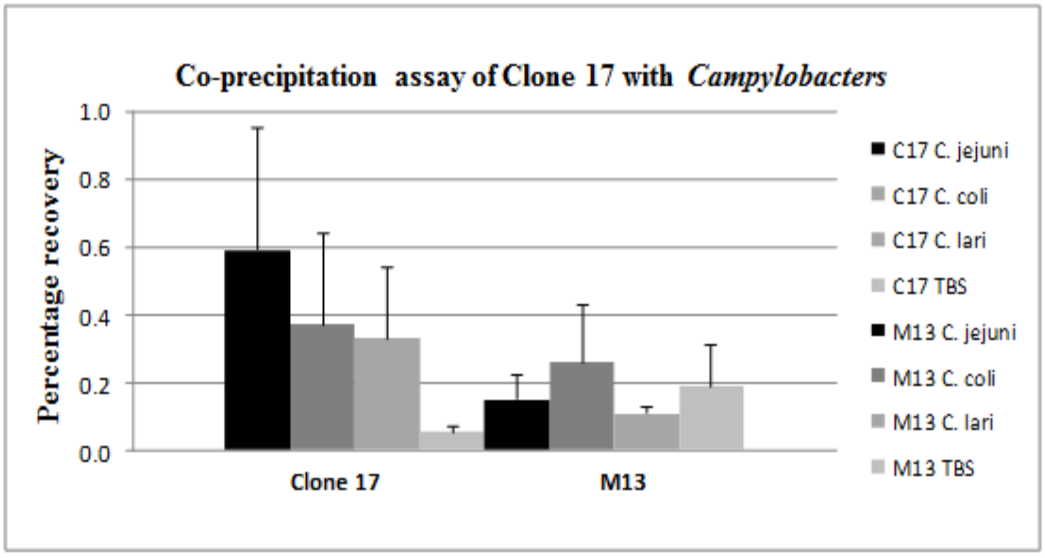

Figure 12. Co-precipitation assay

A co-precipitation assay of clone 17 to was performed to determine the degree of cross-reactivity with closely related *Campylobacters C. coli* and *C. lari*, when assayed in liquid media.

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