EFFECTS OF HOLE SIZE, PRESSURE DIFFERENTIAL, AND SECONDARY PACKAGING ON MICROBIAL INGRESS OF STERILE MEDICAL DEVICE TRAYS

By

Ondrea Kassarjian

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ABSTRACT

EFFECTS OF HOLE SIZE, PRESSURE DIFFERENTIAL, AND SECONDARY PACKAGING ON MICROBIAL INGRESS OF STERILE MEDICAL DEVICE TRAYS

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Nosocomial infections, also referred to as healthcare-associated or hospitalacquired infections (HAIs), are those which hospitalized patients acquire during medical treatment for another condition that were not present or incubating at the time of admission, unless related to a previous admission. These infections are a significant cause of morbidity and mortality that drive up the cost of healthcare in the United States. Despite all of the efforts to prevent nosocomial infections from occurring, they have not been eliminated and are becoming increasingly difficult to treat. It is also difficult determine factors of significance in the causal pathway, such as tracing an infection back to its source. Devices have been cited as sources of infection, both as modes of transmission and reservoirs, but the origin of the pathogens and their transfer to the device are unknown and not widely studied. This raises questions as to whether the devices become contaminated after they are opened, or if their sterile barriers are breached prior to use.

The objective of this research was to examine the effects of hole size, pressure differential, and secondary packaging on microbial ingress of sterile medical device trays.

The methods involved aseptically filling sealed, sterile device packages with a known volume of an appropriate growth medium, exposing the packages to an aerosolized

microbial challenge, incubating the packages, and inspecting for growth. After creating and refining the test techniques, the research explored the impacts of hole size (10 and 100 μ m), pressure differential (0 and -3.78 psi), and secondary packaging (pouches and cartons) on microbial penetration. The specific pressure differential examined simulates an aircraft descending from 8,000 feet or a ground shipment descending from the same elevation.

Hole size, pressure differential, and secondary package type all had a statistically significant effect on microbial penetration of the sterile medical device test trays used in the study.

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INTRODUCTION

Healthcare-associated infections (HAIs) occur in patients who are undergoing treatment for another condition. Consequences of these include increased morbidity, mortality, and costs. HAIs are difficult to treat and, despite the efforts of infection control programs, are not completely preventable. It is also difficult to trace HAIs back to a source.

Medical devices have been identified as sources of infection; however, even when this has been possible, it is still unknown how the device became contaminated. As a result, the creation and maintenance of sterile barrier systems (SBS) and aseptic technique are becoming increasingly important.

The investigation of packaging as a source of infection is limited. Varying results exist as to what types and sizes of defects can be penetrated by pathogenic microorganisms. Additionally, there is a limited amount of research available examining the effects of other variables within the in-use environments, such as pressure differentials and secondary packaging.

This research utilizes microbial challenge methodologies to investigate the effects of hole size, pressure differential, and secondary packaging on the microbial ingress of sterile medical device trays. Desired outcomes include increasing the knowledge base of what facilitates the ability of microorganisms to traverse the sterile barrier. This knowledge can then be applied toward tracing sources of infection and improving package/device designs to maintain the sterile barrier and aid in aseptic technique in an effort to reduce healthcare-associated infections.

LITERATURE REVIEW

2.1 Nosocomial Infections

Nosocomial infections, also referred to as healthcare-associated or hospitalacquired infections (HAIs), are those which hospitalized patients acquire during medical treatment for another condition (Bascetta, Edwards et al. 2008) that were not present or incubating at the time of admission, unless related to a previous admission (Murphy, Whiting et al. 2007). In addition to being extremely costly and a cause of unnecessary suffering (Bascetta, Edwards et al. 2008) nosocomial infections are a significant cause of morbidity and mortality in the United States (Klevens, Edwards et al. 2007). Prolonged hospitalization, the need for ICU care, surgery, or other procedures, reduced activity levels at discharge, and the loss of work (Cosgrove 2006) are among the consequences of HAIs. In fact, for an average 250-bed hospital, these infections result in 2,000 extra days of hospitalization, 20 deaths, and \$1 million in excess costs annually (Jarvis 1996).

Nosocomial infections are becoming more difficult to treat and complete prevention of them has not yet become possible. As such, policy makers and healthcare providers have spent significant attention and resources on programs intended to reduce the prevalence and impact of these infections. Though not emphasized to the same degree as those that concentrate on things like hygienic technique or the use and development of antimicrobials, the creation and maintenance of the sterile barrier system (SBS)¹, as well as its ability to be presented aseptically, are increasingly

important.

¹Sterile Barrier System (SBS): the "minimum package that prevents ingress of microorganisms and allows aseptic presentation of the product at the point of use" (ISO 11607). 2

2.2 Nosocomial Infection Rates

Nosocomial infections rates have been examined and reported by both independent researchers and departments within the US government. The reports vary widely, with some authors suggesting that nosocomial infections rates appear to be improving (Murphy, Whiting et al. 2007), while others suggest that they have increased by 36% in the last 20 years (Schwegman 2008). General rates that have been published range from 1.2% to 51.2% (Table 1).

Not only do the published general infection rates vary, the rates that are specific to type of infection range significantly as well. The Centers for Disease Control and Prevention (CDC) identifies four types of infections as being the most prevalent: urinary tract infections (UTIs), surgical site infections (SSIs), pneumonia or other lung infections, and bloodstream infections (BSIs). Of the total number of nosocomial infections that occur annually in the United States, UTIs are estimated to account for 32%, SSIs for 22%, pneumonia and other lung infections for 15%, and BSIs for 14% (Klevens, Edwards et al. 2007). Ranges of rates published by independent researchers for these types of infections are: 0.6% to 15.5% for pneumonia, 0.27% to 6% BSIs, 2.7% to 7% for SSIs, and 2.39% to 13% for UTIs (Table 1).

Infection Type	Authors and Year	Rate of Infection
	(Craig and Connelly 1984)	8.8%
	(Haley, Culver et al. 1985)	0.6%
Pneumonia	(Craven, Kunches et al. 1988)	9%
	(Leu, Kaiser et al. 1989)	8.6%
	(Kollef 1993)	15.5%
	(Townsend and Wenzel 1981)	4%
Bloodstroom	(Craven, Kunches et al. 1988)	6%
Infoctions (PSIs)	(Haley, Culver et al. 1985)	0.27%
Infections (BSIS)	(Pittet, Tarara et al. 1994)	2.7%
	(Wisplinghoff, Bischoff et al. 2004)	0.6%
	(Haley, Culver et al. 1985)	2.79%
Curraical Cita	(Craven, Kunches et al. 1988)	7%
Surgical Sile	(Kirkland, Briggs et al. 1999)	2.7%
	(Whitehouse, Friedman et al. 2002)	2.8%
Urinary Tract	(Haley, Culver et al. 1985)	2.39%
Infections (UTIs)	(Craven, Kunches et al. 1988)	13%
	(Craven, Kunches et al. 1988)	51.2%
	(Emori and Gaynes 1993)	5.7%
Overall infections	(Fagon, Novara et al. 1994)	5%
Overall infections	(Malone and Larson 1996)	3.9%
	(Richards, Edwards et al. 2000)	6.1%
Care	(Christensen and Jepsen 2001)	17%
	(Graves 2004)	10%
	(Murphy, Whiting et al. 2007)	1.2%

Table 1. Rates of infection by infection type.

2.2.1 Centers for Disease Control and Prevention Rates

The CDC reported that in 2002 there were 1.7 million HAIs which resulted in 176.4 million patient-days. This equates to a rate of 9.3 infections for every 1000 patient-days, or 4.5 infections for every 100 admissions. Of the patients who acquired an infection during this period, adults and children were 93.1% of the population and newborns were 6.9% (Klevens, Edwards et al. 2007).

2.2.2 United States Department of Health and Human Services Rates

In 2010, the US Department of Health and Human Services (DHHS) reported that little progress has been made on eliminating HAIs and that some types have actually increased. According to the DHHS Annual Quality and Disparities Report, the increases in infection rates are as follows: postoperative BSIs by 8%, postoperative catheterassociated UTIs by 3.6%, and selected infections due to medical care by 1.6%. The same data suggests there was no change in the number of central venous catheter BSIs and there was improvement with rates of postoperative pneumonia, which decreased by 12% (U.S. Department of Health and Human Services Agency for Healthcare Research and Quality 2010).

2.2.3 National Nosocomial Infections Surveillance System Rates

The National Nosocomial Infections Surveillance (NNIS) System has also published data which suggests that some types of infections have rates that are decreasing while other types are increasing. The NNIS System was established in 1970 and is composed of the Division of Healthcare Quality Promotion, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, and US Department of Health and Human Services, Atlanta, Georgia. The system collects data from more than 300 hospitals that provide general medical-surgical inpatient services to adults or children within the United States. The surveillance components reported on are: adult and pediatric intensive care units (ICUs), high-risk nurseries (HRNs), and surgical patients (NNIS System 2001; NNIS System 2004).

2.2.3.1 Device-associated Nosocomial Infection Rates in Adult and Pediatric Intensive Care Units

The NNIS has most recently published reports on device-associated nosocomial infections in 2001 and 2004. When comparing these reports, the rates for device-associated UTIs, BSIs, and pneumonia in ICUs have decreased. The average of the pooled means for each infection category suggest that rates decreased for UTIs from 5.9 to 4.9, BSIs from 5.5 to 4.9, and pneumonia from 10.4 to 7.5 (Figure 1) (NNIS System 2001; NNIS System 2004).





2.2.3.2 Device-associated Nosocomial Infection Rates in High-risk Nurseries

When comparing reports published in 2001 and 2004, rates for device-

associated BSIs and pneumonia in high-risk nurseries (HRNs) have also decreased. The

average of the pooled means for each infection category suggest that rates decreased

for BSIs from 6.5 to 5.5 and pneumonia from 3.5 to 2.3 (Figure 2) (NNIS System 2001; NNIS System 2004).



Figure 2. Average of pooled means of NNIS System device-associated infection rates for bloodstream infections (BSI) and pneumonia in HRNs (NNIS System 2001; NNIS System 2004).

2.2.3.3 Surgical Site Infection Rates

While device-associated UTIs, BSIs, and pneumonia rates have all decreased,

surgical site infection rates have not had the same improvement according to NNIS

System data. Comparisons of the averaged pooled means from 2001 and 2004 NNIS

System reports suggest that the rate for surgical site infections (SSIs) has increased from

2.53 to 2.73 (Figure 3) (NNIS System 2001; NNIS System 2004).



Figure 3. Average of pooled means of NNIS System surgical site infection rates (NNIS 2001 and 2004).

The majority of nosocomial infections occurs within only four categories (infection types) and affects all age populations, including newborns. Despite the variation of infection rates, and whether the reports indicate they are increasing or decreasing, they are still occurring. Knowledge exists on sterile environments and infection control programs and procedures are in place, but again, the infections continue to happen. This raises questions as to what the unidentified causes of these infections are and why they have not been eliminated.

2.3 Causes of Nosocomial Infections

The word "nosocomial" is derived from the Greek and Latin words for hospital. In Roman times, hospital orderlies were referred to as nosocomi and hospital infections were believed to be caused by miasmi, which is a vaporous exhalation of bad air (Wilcox 2003). Now, obviously, there is a much greater understanding of the real causes of nosocomial infections; they are most frequently associated with high-risk medical interventions such as surgical procedures and the use of invasive devices (Emori and Gaynes 1993). The sources of infection and modes and rates of transmission can vary in relation to the hospital environment, how well hospital employees adhere to infection control procedures, and the immune statuses of patients (Schwegman 2008).

2.3.1 Hospital Environment

Hospitals are "more likely to spread infections than to stop them" and patients are now more scared of catching an infection than going under the knife" (Cole 2008). This is because bacteria are infecting patients despite the efforts of procedural techniques and the maintenance of a sterile environment (Schierholz and Beuth 2001). Not only are microorganisms transmitted through direct contact between people, but contaminated intermediate objects serve as a means of indirect contact. Non-sterile medical devices including blood pressure meters, thermometers, and stethoscopes have all served in the spread of nosocomial infections (Schwegman 2008). Nosocomial pathogens are also able to be transmitted through both wet media and dry surfaces. *Staphylococci* are able to survive under dry conditions for long periods of time; therefore, common items within a hospital environment such as furniture, equipment, instruments, and even sterile packaging can become reservoirs for microorganisms (Dietze, Rath et al. 2001).

2.3.2 Adherence to Infection Control Procedures

Extrinsic factors that contribute to the spread of HAIs include the practices of individuals and hospitals (Emori and Gaynes 1993). Hospital personnel can fail to adhere to infection control procedures for hand hygiene and surgical hand antisepsis,

intravenous site preparation and selection, dressing changes, and barrier protection (Gilmore 2003); barrier protection is defined to include proper use of gloves, protective eyewear, gowns, and masks (Emori and Gaynes 1993). Other factors outside the point of care include isolation facilities, bed occupancy, staffing levels, education and training (Cole 2008), antimicrobial use (Weinstein 1998), and the care of devices (Stone, Larson et al. 2002).

Most of these extrinsic factors are not directly related to packaging; however, the care (and use) of devices has the potential to be impacted. Adherence to infection control procedures is dependent on the users of sterile devices and the environment they are working in. Understanding how sterile barriers fail can be applied to improve sterile barrier maintenance within these environments. Additionally, improving package/device designs which aid in aseptic technique can also facilitate better adherence to infection control procedures.

2.3.3 Patient Immune Status

Risk of infection can be decreased with shorter lengths of stay in the hospital and by reducing inpatient surgeries altogether; risk increases with longer lengths of stay and in patients who are elderly or critically ill (Malone and Larson 1996). Immunocompromised patients are at a greater risk of infection due to their age, underlying diseases, and the medical or surgical treatments they receive. Even more alarming is that the patient population in the hospital is becoming more immunocompromised because the sickest patients remain in the hospital while the others receive outpatient care (Weinstein 1998). Not only are these patients the most

susceptible, but they contribute to the spread of infection as well; however, HAIs should not be an acceptable and expected outcome of treating an older, sicker patient population (Murphy, Whiting et al. 2007). Patients who are immunocompromised cannot be controlled or changed, but the environment around them, and the devices used to treat them, can be.

2.3.4 Medical Devices

Invasive medical devices have been examined for their role in nosocomial infections. From 1992 to 1998, 54% of nosocomial infections occurred in surgical patients with most of them linked to a particular invasive medical device. Central intravenous lines were associated with 87% of BSIs, mechanical ventilators were associated with 83% of nosocomial pneumonias, and catheters were associated with 97% of UTIs (Richards, Edwards et al. 2000). These types of devices allow pathogens easy entry into the body (Wenzel 2000) by serving as a pathway from the environment or one part of the patient's body to another. They also serve as reservoirs where microbes colonize, protected from the patient's immune defenses; for instance, bacteria colonize on catheters (Hu, Veenstra et al. 2004). As a result, the higher rates of pneumonia, UTIs, and BSIs have been linked to the use of these devices both during and following surgeries (Emori and Gaynes 1993).

Implanted devices have also been implicated as source of 45% of nosocomial infections (Schierholz and Beuth 2001). Here, the use of implant devices either damages or invades epithelial or mucous barriers in the patient. Again, they serve as a reservoir for microbial colonization and are protected from or impair the patient's immune

defenses which results in chronic infection or tissue necrosis. Implant infections are also influenced by the patient's immune and cellular defenses. Interestingly, the ability of bacteria to adhere to a device is dependent on the device material's topography, trace chemicals, and ionic and glycoprotein sequences and, despite adherence to sterile procedures for implants, device-associated infections are still occurring (Schierholz and Beuth 2001).

2.4 Consequences of Nosocomial Infections

There are a multitude of consequences that result from nosocomial infections. These include increased lengths of stay in the hospital for the infected patients, which drive up costs for both the patients and hospitals. There is also a series of socioeconomic costs, which are difficult to measure and affect both the patients and the people associated with them, such as family and employers. The most severe consequence, however, is mortality that is attributable to these infections.

2.4.1 Length of Stay

Nosocomial infections affect both the intensity and the duration of care. Excess lengths of stay (LOS) are additional, beyond the usual LOS (and costs) for the original admissions, and would be avoided if the infections did not occur (Murphy, Whiting et al. 2007). The risk of death has also been indicated to increase with duration of stay due to the increased risk of acquiring a fatal infection during hospitalization (Fagon, Novara et al. 1994). Patients with HAIs tend to have longer LOS while sicker patients who require longer LOS are at an increased risk of acquiring an infection. This has led some authors to questions whether nosocomial infections are the cause of significant extra LOS and

costs, or if they are relatively inexpensive and inevitable consequences of long and expensive hospitalizations (Kilgore, Ghosh et al. 2008).

Nosocomial infections can create a significant amount of excess patient days in the hospital that can range from days to weeks per infection. In 1985, it was estimated that HAIs created an excess 7.5 million patient days nationwide (Murphy, Whiting et al. 2007). This equates to 20,548 years of unintended hospitalization for the American public! In 2005, the Pennsylvania Healthcare Cost Containment Council (PHC4) reported that they had approximately 1.9 million hospital admissions without HAIs and 24,000 with HAIs. The average lengths of stay were less than 5 days and 23 days, respectively. On average, the patients with infections had to spend an additional 18 days hospitalized!

Published studies indicate ranges of excess LOS attributable to nosocomial infections for the four main types of infections, infections overall, and for patients who acquire multiple infections (Table 2). Excess LOS for pneumonia, BSIs, SSIs, and UTIs range from 4.7 to 30 days, 7 to 25.8 days, 7 to 14 days, and 1 to 12.5 days, respectively. Overall excess LOS for infections due to medical care ranges from 4 to 18 days. Additionally, those patients who have multiple infections have excess LOS of approximately 35.9 days.

Infection Type	Authors and Year	Excess LOS (days)
	(Craig and Connelly 1984)	7.7
	(Haley, Culver et al. 1985)	6
Droumonio	(Leu, Kaiser et al. 1989)	7
Prieumonia	(Jarvis 1996)	6.8 - 30
	(Heyland, Cook et al. 1999)	4.7
	(Warren, Shukla et al. 2003)	25
	(Murphy, Whiting et al. 2007)	25.7
	(Townsend and Wenzel 1981)	20
	(Haley, Culver et al. 1985)	7
Dlaadstraam	(Pittet, Tarara et al. 1994)	24
BIOOUSTREAM	(Jarvis 1996)	7 - 21
	(DiGiovine, Chenoweth et al. 1999)	10
	(Zhan and Miller 2003)	10.89
	(Wisplinghoff, Bischoff et al. 2004)	7.5 - 25
	(Murphy, Whiting et al. 2007)	25.8
	(Haley, Culver et al. 1985)	7
Surgical Sita	(Jarvis 1996)	7 – 8.2
Infoctions (SSI)	(Kirkland, Briggs et al. 1999)	12
	(Whitehouse, Friedman et al. 2002)	14
	(Murphy, Whiting et al. 2007)	7.5
Uripany Tract	(Givens and Wenzel 1980)	2.4
Unitary Tract	(Haley, Culver et al. 1985)	1
	(Jarvis 1996)	1 - 4
	(Murphy, Whiting et al. 2007)	12.5
	(Haley, Culver et al. 1985)	4
	(Malone and Larson 1996)	6.5 - 7
Overall Infections	(Richards, Edwards et al. 2000)	2.3
Due to Medical	(Zhan and Miller 2003)	9.58
Care	(Murphy, Whiting et al. 2007)	18
	(Kilgore, Ghosh et al. 2008)	5.4
	(Schwegman 2008)	7.4 - 9.4
Multiple Infections	(Murphy, Whiting et al. 2007)	35.9

Table 2. Excess lengths of stay due to nosocomial infection by infection type.

2.4.2 Patient Costs

Nosocomial infections drive up patient costs due the additional treatments and duration of care, which also contributes to the high cost of healthcare in the United States. Total healthcare costs and their proportion of the gross domestic product (GDP) have been increasing in the United States and healthcare spending is expected to outpace economic growth by 1.9 percentage points annually (Keehan, Sisko et al. 2008). In 2002, total US healthcare costs accounted for approximately 13.5% of the GDP, or \$1.1 trillion (Stone, Larson et al. 2002). In 2007, total healthcare spending in the US was \$2.4 trillion, or \$7,900 per person, and 17% of the GDP. In 2009, it increased to \$2.5 trillion, or 17.6% GDP (National Coalition on Health Care 2009). By 2017, it is expected to increase to \$4.3 trillion, or 20% of the GDP (National Coalition on Health Care 2009).

Estimates of excess healthcare costs due to nosocomial infections are reported to be between \$4.5 and \$5.7 billion annually (Soule 2008); however, estimates are difficult to obtain, as the underlying condition of each infected patient affects the duration, type, and costs of their treatment (Anderson, Kirkland et al. 2007). It is also believed that many figures are underestimated, since they do not always include the additional costs of outpatient care, in-home care, readmission (Kirkland, Briggs et al. 1999), additional procedures, physical therapy, and intravenous antibiotic therapy (Whitehouse, Friedman et al. 2002).

Scott (2009) estimates total annual costs (in 2007 US \$) for inpatient services in US hospitals associated with specific sites of HAIs as:

- Ventilator-associated pneumonia (VAP): \$780 million to \$1.5 billion
- Central line-associated bloodstream infection (CLABSI): \$590 million to \$2.68 billion
- Surgical site infection (SSI): \$3.2 to \$10 billion
- Catheter-associated urinary tract infection (CAUTI): \$340 million to \$450 million

• Clostridium difficile-associated disease (CID): \$1.01 to \$1.62 billion (Scott 2009)

Published studies indicate ranges of attributable costs (services provided and billed to a patient that were caused by an HAI (Murphy, Whiting et al. 2007) by type of infection and overall (Table 3). Estimates of attributable costs per patient range from: \$4,947 to \$25,072 for pneumonia, \$3,061 to \$52,727 for BSIs, \$2,734 to \$27,969 for SSIs, and \$558 to \$1,006 for UTIs. Estimates of overall attributable costs per patient due to medical care range from \$1,833 to \$38,656.

Infection Type	Authors and Year	Attributable Costs
	(Haley, Culver et al. 1985)	\$4,947
	(Stone, Larson et al. 2002)	\$17,677
Pneumonia	(Warren, Shukla et al. 2003)	\$11,897
	(Stone, Braccia et al. 2005)	\$9,969
	(Anderson, Kirkland et al. 2007)	\$25,072
	(Haley, Culver et al. 1985)	\$3,061
	(Pittet, Tarara et al. 1994)	\$40,000
	(DiGiovine, Chenoweth et al. 1999)	\$34,508
Bloodstream	(Stone, Larson et al. 2002)	\$38,703
Infections (BSIs)	(Zhan and Miller 2003)	\$52,727
	(Hu, Veenstra et al. 2004)	\$5,374 - \$22,939
	(Stone, Braccia et al. 2005)	\$36,441
	(Anderson, Kirkland et al. 2007)	\$23,242
	(Haley, Culver et al. 1985)	\$2,734
	(Kirkland, Briggs et al. 1999)	\$5,038
Surgical Sita	(Hollenbeak, Murphy et al. 2002)	\$14,211 - \$20,103
Infoctions (SSIs)	(Stone, Larson et al. 2002)	\$15,646
	(Whitehouse, Friedman et al. 2002)	\$27,969
	(Stone, Braccia et al. 2005)	\$25,546
	(Anderson, Kirkland et al. 2007)	\$10,443
	(Givens and Wenzel 1980)	\$558
Urinary Tract	(Haley, Culver et al. 1985)	\$593
Infections (UTIs)	(Stone, Braccia et al. 2005)	\$1,006
	(Anderson, Kirkland et al. 2007)	\$758

Table 3. Attributable costs due to nosocomial infections by infection type.

Table 3 (cont'd).

Overall Infections Due to Medical Care	(Haley, Culver et al. 1985)	\$1,833
	(Stone, Larson et al. 2002)	\$13,973
	(Zhan and Miller 2003)	\$38,656
	(Murphy, Whiting et al. 2007)	\$8,832
	(Kilgore, Ghosh et al. 2008)	\$12,197

2.4.3 Hospital Costs

Nosocomial infections also consume hospital resources as a result of the additional diagnostic and therapeutic interventions they require (Graves 2004). The overall direct cost of HAIs for US hospitals has been estimated between \$28.4 and \$45 billion annually (Scott 2009). HAIs have also been estimated to reduce overall net inpatient margins by \$1,779 (Jarvis 1996) to \$5,018 per infected patient (Murphy, Whiting et al. 2007).

Malpractice lawsuits add to the mounting costs. The average 250-bed hospital (or its malpractice carrier) spends between \$300,000 and \$1 million annually defending malpractice lawsuits, not including the settlement and judgment amounts. Nosocomial infections are listed as the eighth leading malpractice claim. Glabman indicates the following:

- 1. Medication Errors
- 2. Diagnosis Failures
- 3. Negligent Supervision
- 4. Delayed Treatment
- 5. Failure to Obtain Consent
- 6. Lack of Proper Credentialing or Technical Skill

7. Unexpected Death

- 8. latrogenic Injury, Nosocomial and Wound Infections, Fractures
- 9. Pain and Suffering, Emotional Distress
- 10. Lack of Teamwork, Communication (Glabman 2004)

2.4.4 Socioeconomic Impact

Socioeconomic costs are composed of not only direct medical costs for treatment, but also indirect costs related to loss of productivity for the patient and nonmedical costs and intangible costs related to diminished quality of life. While the direct medical costs are more easily quantified, the indirect and intangible costs consist of a series of rippling consequences that are often overlooked and difficult to calculate. Indirect costs include not only lost time, wages, and productivity for the patient, but also for their family members who take time off from work to travel to visit the patient and become caregivers. Additionally, patient morbidity also reduces leisure time and can lead to death. Intangible costs are those that have no monetary value but can have a permanent impact on the patient and their loved ones. These include psychological pain and suffering and changes in social functioning and the ability to perform daily routines (Scott 2009).

2.4.5 Mortality

Nosocomial infections are a significant cause of mortality in the United States (Klevens, Edwards et al. 2007). They are fourth leading cause of death in the US following heart disease, cancer, and stroke (Jarvis 1996). In 2002, there were 1.7 million HAIs resulting in 98,987 deaths (Klevens, Edwards et al. 2007). It is estimated that

100,000 deaths occur annually in the US as a result of nosocomial infections (Murphy, Whiting et al. 2007).

As with the other estimates reviewed, reported attributable mortality rates vary widely and depend largely on the type of infection (Table 4). In 1994, the literature stated that mortality was highly probable for pneumonia, doubtful for bacteremia (bloodstream infections), and uncertain for urinary tract infections (Fagon, Novara et al. 1994); however, further review of the literature indicates that pneumonia and bloodstream infections have high rates of attributable mortality while UTIs have not yet had attributable mortality rates linked to them in a publication. The ranges of attributable mortality rates for patients with infections are: 5.8% to 60% for pneumonia, 4.4% to 35% for BSIs, and 3.4% to 4.3% for SSIs. Attributable mortality rates used to infections in surgical and medical ICUs are estimated at 7% while overall nosocomial infection mortality rates range from 4.31 to 5%.

Infection Type	Authors	Attributable Mortality
Pneumonia	(Craig and Connelly 1984)	14.7%
	(Leu, Kaiser et al. 1989)	6.8%
	(Kollef 1993)	28.7%
	(Jarvis 1996)	6.8 - 30%
	(Heyland, Cook et al. 1999)	5.8%
	(Warren, Shukla et al. 2003)	16%
	(Murphy, Whiting et al. 2007)	20-60%

Table 4. Attributable mortality rates due to nosocomial infections by infection type.

Bloodstream Infections (BSIs)	(Townsend and Wenzel 1981)	21%
	(Smith, Meixler et al. 1991)	29.5%
	(Pittet, Tarara et al. 1994)	35%
	(Jarvis 1996)	16.3 - 35%
	(DiGiovine, Chenoweth et al. 1999)	4.4%
	(Zhan and Miller 2003)	21.92%
	(Wisplinghoff, Bischoff et al. 2004)	27%
	(Murphy, Whiting et al. 2007)	20 - 60%
Surgical Site	(Kirkland, Briggs et al. 1999)	4.3%
Infections (SSIs)	(Whitehouse, Friedman et al. 2002)	3.4%
Infections in Surgical and Medical ICUs	(Craven, Kunches et al. 1988)	7%
Overall Infections	(Zhan and Miller 2003)	4.31%
Due to Medical Care	(Schwegman 2008)	5%

Table 4 (cont'd).

2.4.6 Staph Infections

Staphylococcus aureus is reported as the most common cause of nosocomial infection in the United States (Cosgrove, Qi et al. 2005); as a result, researchers often study its impact as related to the previously discussed consequences. Staph infections, including methicillin-resistant *staphylococcus aureus* (MRSA), occur most frequently in hospitals and healthcare settings in patients who have already weakened immune systems (Division of Healthcare Quality Promotion 2010). They are the primary cause of nosocomial pneumonia and surgical site infections and the second leading cause of bloodstream infections (Cosgrove, Qi et al. 2005). Wisplinghoff et al. estimate that 20% of bloodstream infections in hospital settings are caused by *S. aureus* (Wisplinghoff, Bischoff et al. 2004).

Some researchers have studied how nosocomial *S. aureus* infections impact economic burden, length of stay, and mortality. Rubin et al. estimated that *S. aureus*

infections double the length of stay, number of deaths, and costs of typical hospitalizations. Researchers estimated the direct costs to be \$28,800 per patient with a mortality rate of 10.1%. When *S. aureus* infections were methicillin-resistant, treatment costs increased to \$31,400 per patient. Comparatively, a typical hospital stay (without infection) cost \$13,263, had a length of stay of 9 days, and had a mortality rate of 4.1% (Rubin, Harrington et al. 1999).

Noskin et al. also evaluated *S. aureus* economic burden and mortality rates and compared inpatient stays with surgical stays. Researchers estimated that the cost per infected patient was \$37,352 for all inpatient stays and \$40,637 for all surgical stays. Mortality rates were approximately equal, with a rate of 5.6% for all inpatient stays and 5.5% for surgical stays (Noskin, Rubin et al. 2007).

2.4.6.1 MRSA

Multidrug-resistant organisms (MDROs) are becoming a greater concern for HAIs because they are becoming more prevalent. They can cause any type of HAI including skin infections, BSIs, pneumonia, SSIs, and UTIs. They also exacerbate the consequences of HAIs leading to even longer hospital stays, higher mortality rates, and higher treatment costs because they are more difficult to treat (Bascetta, Edwards et al. 2008). In fact, antimicrobial resistant infections are estimated to cost approximately \$6,000 to \$30,000 more to treat than antimicrobial susceptible organisms (Cosgrove 2006).

Methicillin-resistant *staphylococcus aureus* (MRSA) is considered an MDRO and is resistant to methicillin, oxacillin, penicillin, and amoxicillin (Division of Healthcare Quality Promotion 2010). MRSA is one of the most common antimicrobial resistant

pathogens and occurs most frequently in patients who have undergone invasive procedures or have weakened immune systems and are treated in hospitals and healthcare facilities (Williams 2008). It reportedly causes three of the top four categories of nosocomial infections: bloodstream, surgical site, and pneumonia (Division of Healthcare Quality Promotion 2007). The main mode of transmission to patients is through healthcare workers' hands which can become contaminated and transport microorganisms between patients, personnel, devices, other items, and surfaces (Division of Healthcare Quality Promotion 2007).

One intermediate object that can serve as a pathway for microorganisms is cell phones. In 2009, Ulger et al. published a study on health care workers' cell phone contamination. Two hundred participants and their cell phones were cultured and nosocomial pathogens were found on several of the phones. Researchers found that 94.5% of healthcare workers' cell phones had bacterial contamination and that rates were similar between hands and phones. Fifty-two percent of the *S. aureus* strains isolated from the phones and 37.7% from the healthcare workers' hands were MRSA. Researchers concluded that cell phones may be a source of nosocomial infections in hospitals (Ulger, Esen et al. 2009).

MRSA infections in US intensive care units increased from 35.5% to 64.4% between 1992 and 2003 (Klevens, Edwards et al. 2006). The Division of Healthcare Quality Promotion reported that in 1974, MRSA represented 2% of all staph infections and increased to 22% by 1995, and 63% by 2004 (Figure 4) (Division of Healthcare
Quality Promotion 2007). Clearly, the resistance of this organism is a growing cause for concern.



Figure 4. Percent of methicillin-resistant staph infections (Division of Healthcare Quality Promotion 2007).

The national impact that MRSA will have is unknown (Kuehnert, Hill et al. 2005). Evidence does suggest, however, that resistant infections result in higher mortality rates (Rubin, Harrington et al. 1999). Stone et al. reported that the attributable cost of a MRSA infection is \$35,367 (Stone, Larson et al. 2002). MRSA has also been estimated to add 2.7 million extra days in the hospital annually (Schwegman 2008).

Klevens et al. examined healthcare-associated infections in 16.5 million patients, or approximately 5.6% of the US population. There were 8,987 cases of MRSA reported during the surveillance. Of these, 92.4% of the patients were hospitalized, 17.8% died, and 12.9% developed recurrent invasive infections. After adjusting for age, race, and sex to the US population, researchers estimated that there were 94,360 invasive MRSA infections in the United States in 2005 that resulted in 18,650 deaths (Klevens, Morrison et al. 2007). The CDC estimates that 86% of MRSA infections were healthcare associated and uses these figures as an annual estimate of the prevalence and impact of MRSA (Division of Healthcare Quality Promotion 2007).

2.4.6.1.1 Impact of MRSA

Researchers have published reports comparing the impacts of drug resistant and drug susceptible *S. aureus* infections, MRSA and MSSA, respectively. Cosgrove et al. compared the impacts of methicillin-susceptible *staphylococcus aureus* (MSSA) and MRSA on length of stay, mortality, and costs. Researchers examined data collected from 348 patients with *S. aureus* bloodstream infections; 73% contracted MSSA while 28% contracted MRSA. Cosgrove et al. concluded that the median attributable impact of MRSA, compared to MSSA, included an additional 2 days LOS (for patients who survived) (p = .045), hospital charges of \$6,916 per patient (p = .008), and hospital costs of \$3,836 per patient. Mortality rates were similar (p = .53) for both MSSA (19.8%) and MRSA (22.9%) (Cosgrove, Qi et al. 2005).

Engemann et al. compared the impacts of MSSA and MRSA on length of stay, mortality, and costs for patients who underwent a surgical procedure and subsequently tested positive for *S. aureus*. There were 479 usable patients: 40% were control subjects (uninfected patients who underwent similar procedures), 35% had a MSSA SSI, and 25% had a MRSA SSI. Researchers reported that the median LOS after surgery was 5, 14, and 23 days, respectively. The median LOS after infection was 10 days for MSSA and 15 days for MRSA. The mortality rates were 2.1% for the control group, 6.7% for MSSA patients, and 20.7% for MRSA patients. Researchers concluded that the adjusted mean attributable excess hospital charges per patient were \$13,901 for MRSA compared with MSSA and \$41,274 for MRSA compared with the control group (Engemann, Carmeli et al. 2003).

2.4.6.2 Staphylococcus epidermidis

In 2002, Vuong and Otto identified *Staphylococcus epidermidis* as becoming the most important cause of nosocomial infections. It is linked with bloodstream, cardiovascular, eye, ear, nose, and throat infections, typically in patients who are already compromised. While it is a normal bacterial flora that colonizes on the skin and mucous membranes of humans, it can change from being innocuous to infectious through the formation of biofilm (the main virulence factor) on indwelling intravascular catheters and medical devices. Treatment is difficult because the biofilm protects against attacks from the immune system and creates an impermeable barrier to many antibiotics. Approximately 80% of nosocomial *S. epidermidis* infections are resistant to methicillin and other antibiotics. When these infections occur, the device usually has to be removed and replaced (Vuong and Otto 2002).

2.5 Prevention of Nosocomial Infections

Opposing philosophies exist regarding HAIs. Some believe that "many infections are inevitable, although some can be prevented" while others believe "each infection is potentially preventable until proven otherwise" (Gerberding 2002). Either way, however, "prevention is better than cure" because it saves money and prevents pain, suffering, and death (Harbath, Sax et al. 2003).

Nosocomial infections are a preventable cause of morbidity and mortality.

Harbath estimates that the range of preventable nosocomial infections is between 10-70% and at least 20% of are "probably preventable" (Harbath, Sax et al. 2003). Jarvis estimates that full implementation of current infection control recommendations would reduce approximately one third of preventable nosocomial infections (Jarvis 1996).

In addition to the noble goals of reducing morbidity and mortality, most prevention strategies are likely cost-attractive as well (Harbath, Sax et al. 2003). In the US, it has been estimated that a 20% prevention rate of HAIs would save between \$5.7 to \$6.8 billion annually, and \$25 to \$31.5 billion would be saved if 70% of HAIs were prevented (Scott 2009).

2.5.1 Infection Control Programs

In 1976, the Joint Commission on Accreditation of Healthcare Organizations published infection control standards. These standards demanded the creation of administrative and financial support for infection control programs. Early data, published by the CDC in 1985, examined the efficacy of nosocomial infection control. The report indicated that rates were reduced by one third when hospitals had an effective epidemiologist, one infection control practitioner for every 250 beds, active surveillance methods, and ongoing control efforts (Weinstein 1998).

CDC recommendations have evolved to now encompass 13 guidelines (based on scientific evidence) for infection control and prevention for hospitals. Examples include:

- Guideline for Prevention of Catheter-associated Urinary Tract Infections (1981)
- Guideline for Prevention of Surgical Site Infection (1999)

- Guidelines for Environmental Infection Control in Health-Care Facilities (2003)
- Guidelines for Preventing Health-Care-Associated Pneumonia (2003)
- Management of Multidrug-Resistant Organisms in Healthcare Settings (2006)
- Guideline for Isolation Precautions: Preventing Transmission of Infectious Agents in Healthcare Settings (2007)

These guidelines include nearly 1,200 implementation practices to prevent HAIs, such as using alcohol-based hand sanitizers, isolating infected patients, properly sterilizing equipment, treating patients with antibiotics prior to surgeries, and annually vaccinating healthcare workers for the flu (Bascetta, Kohn et al. 2008). Notably, there is no reference to packaging or sterile barriers and the transmission of HAIs. Healthcare workers seem to trust that the devices are sterile at the point of use and are not suspicious otherwise.

2.5.1.1 Antimicrobial Use and Resistance

A significant area of emphasis in infection control programs is the appropriate use of antimicrobials. This emphasis is largely the result of reports that suggest that a severe threat exists for a post-antibiotic era and aggressive antibiotic control programs are needed to prevent this (Weinstein 1998). The World Health Organization (WHO) reported in 2002 that some diseases will have no effective therapies within the next ten years and issued the following statement: "Most alarming of all are diseases where resistance is developing for virtually all currently available drugs, thus raising the spectre of a post anti-biotic era" (World Health Organization 2002). One of the causes of the rapid increase in resistance is from widespread overuse of antibiotics by medical professionals. Hospitals are identified as critical contributors to antimicrobial resistance because they practice routine intensive antimicrobial use, contain already susceptible patients, and have occurrences of cross-infection. These combined factors have resulted in nosocomial infections which are highly resistant to treatment and are not only expensive to control, but extremely difficult to eradicate as well (World Health Organization 2002). Additionally, one half of the antibiotics used in the US to treat humans are also used to treat domestic animals. This concurrent use enhances selection for drug-resistant microbes, further exacerbating the problem of resistance (Pimentel, Tort et al. 1998).

Due to the development of antimicrobial resistance, antibiotic stewardship is becoming increasingly important. Antibiotic control methods include prescription tracking and restricting the use of certain antibiotics (Bascetta, Edwards et al. 2008); however, prompt and appropriate antimicrobial therapy is also required when infections do develop (Gerberding 2002).

To treat infections, inexpensive and effective first-line drugs are typically used; however, these drugs tend to be the treatments that microorganisms are becoming resistant to. When first-line drugs fail, second- or third-line drugs are implemented, but these are almost always more expensive (sometimes by 100 times) and can also be more toxic (World Health Organization 2002).

When antibiotic therapies fail, the patients and American society both experience additional burdens. Healthcare system expenses increase (Division of

Healthcare Quality Promotion 2007) and infected patients are more likely to experience prolonged illnesses that result in increased lengths of stay, costs, and risk of death (World Health Organization 2002; Division of Healthcare Quality Promotion 2007). Additional factors that may contribute to these consequences include increased toxicity, improper dosing, and delay in treatment (Cosgrove 2006).

Antimicrobial resistance develops when microbes adapt as a result of the drug treatment used; the microorganisms are forced to either adapt or die. Those that adapt then carry genes for resistance and can no longer be killed with standard antimicrobial treatments. Bacteria are efficient in creating resistance because they can multiply quickly and pass on their resistance genes during replication. Additionally, resistant bacteria can pass on their resistance genes through a process called conjugation; here, plasmids carrying the genes jump from one bacterium to another (World Health Organization 2002).

The NNIS System, referenced previously, has reported on antimicrobial resistance of pathogens associated with nosocomial infections in ICUs. Researchers suggest that antimicrobial resistance is continuing to increase for 8 of the 9 drug/pathogen combinations reported (Figure 5) (NNIS System 2001; NNIS System 2004).



Figure 5. Comparison of NNIS System antimicrobial resistance rates in 2001 and 2004 (NNIS System 2001; NNIS System 2004).

While antimicrobial resistance has been increasing, the rate at which it has been occurring appears to have slowed. When comparing reports, the rate at which resistance progressed has decreased in 8 of the 9 drug/pathogen combinations (Figure 6) (NNIS System 2001; NNIS System 2004).



Figure 6. Comparison of NNIS System increase in antimicrobial resistance in 2001 and 2004 (NNIS System 2001; NNIS System 2004).

In 2008, Klevens et al. used the NNIS System data to compare antimicrobialresistant infection levels for three specific types of infection between the periods of 1990-1994 (207 hospitals) and 2000-2004 (292 hospitals). A significant increase (p<.001) in the proportion of antimicrobial resistant infections was found for methicillinresistant *S. aureus* bloodstream infections (MRSA BSI), ceftazidime-resistant *P. aeruginosa* pneumonia (CRPA pneumonia), and ciprofloxacin-resistant *E. coli* urinary tract infections (CREC UTI). Their resistance rates increased from 27% to 54.1%, 16.6% to 22.7%, and 0.9% to 9.8%, respectively (Figure 7) (Klevens, Edwards et al. 2008).



Figure 7. Comparison of antimicrobial-resistance rates between 1990-1994 and 2000-2004 (Klevens, Edwards et al. 2008).

2.5.1.2 Other Prevention Measures

Appropriate antibiotic use and hand hygiene are significant parts of infection control programs. Those related to hands include general washing, antisepsis, and hygiene techniques, choice of hygiene agents, and surgical hand antisepsis (Schwegman 2008). Other infection prevention and control measures include:

- Continual improvement of surveillance so data is increasingly representative and ensuring the surveillance uses are valid (Weinstein 1998)
- Contact precautions such as the use of gloves, gowns, and masks (Bascetta, Edwards et al. 2008)
- Enhanced environmental cleaning (Bascetta, Edwards et al. 2008)
- Appropriate handling of laundry (Division of Healthcare Quality Promotion 2007)
- Patient placement and transport (Division of Healthcare Quality Promotion 2007)

Appropriate handling of patient care equipment and instruments/devices
(Division of Healthcare Quality Promotion 2007).

As the care of devices was previously discussed as a potential source of infection, here again, there is potential for infection prevention by gaining a greater understanding of how sterile barriers fail and improving package/device designs which aid in aseptic technique.

2.5.2 Device and Packaging Design

Improving the design of invasive devices is another option that is particularly interesting and potentially quite innovative. "Given the choice of improving technology or improving human behavior, technology is the better choice" (Weinstein 1998). The theory behind this is, given the choice of changing human behavior (improving aseptic technique or adhering to procedure) or designing a better device, the device will be the more successful option (Weinstein 1998).

Devices with better functionality may have the potential to reduce the rates of HAIs. One approach is to use materials that mitigate the colonization of microorganisms. Another approach is to design devices which facilitate aseptic technique. Again, this is an opportunity to change the environment so that the user is successful in executing infection control procedures. Taking this a step further, improving the design of device packaging has the potential for the same benefits. Here, the device and its package could work in tandem, optimizing their functionality, so that the package aids in device placement (Allen 2010).

2.5.3 Successful Prevention Initiatives

There is evidence that infection control programs are working. In a 500-bed study institution in Arkansas, the introduction of the OSHA Control Plan significantly lowered (p<.001) nosocomial infection rates from an average of 3.9% to 2.6% during a three-year study (1991-1993) through the concomitant increase in glove use and widespread use of barrier hand foam (Malone and Larson 1996).

There is also evidence that even small investments aimed at prevention have been successful in reducing both the number of infections and associated costs. In 2004, the Jewish Health Foundation and Pittsburg Regional Health Initiative implemented an evidence-based prevention measure across 40 hospitals to reduce central line associated bloodstream infections (CLABs) which resulted in an overall reduction of 63% (results were audited by the CDC). One participant in the initiative, Allegheny General Hospital, also reduced ventilator-associated pneumonias (VAPs) by 82%. Their two- and one-year efforts to eliminate CLABs and VAPs, respectively, resulted in a total cost savings of \$2.2 million. The initial cost to obtain that savings was only about \$35,000 (Murphy, Whiting et al. 2007).

A similar outcome was noted when BJC Healthcare, a 13-hospital, non-profit healthcare system based in St. Louis, MO increased resources to eliminate HAIs from 2000-2004. Their excess costs were estimated at \$8.2 million for coronary artery bypass graft (CABG) and spinal surgical site infections, bloodstream infections, and ventilator associated pneumonia. They invested \$350,000 across the system and individual hospitals within it invested an additional \$50,000-\$150,000 to increase the number of

staff dedicated to infection prevention and/or medical direction during that period. This reduced CABG SSIs by 18%, spinal SSIs by 61%, BSIs by 82%, and VAP by 52% resulting in a cost savings of \$2.5 million (Murphy, Whiting et al. 2007).

In addition to the cost savings, it is likely that these reduced rates of infection also resulted in lower mortality rates, which raises the question, "What is the cost of savings lives?" However, despite the focused efforts and improvements of infection control programs, nosocomial infections still have not been eliminated. This suggests that there are unknown contributors to HAIs which require investigation and supports why examining the sterile barrier as a potential source of infections is necessary.

2.6 Packaging

Despite all of the efforts to prevent nosocomial infections from occurring, they have not been eliminated and are becoming increasingly difficult to treat. It is also difficult determine factors of significance in the causal pathway, such as tracing an infection back to its source. There are widely accepted surveillance methods for tracking and tracing nosocomial infections, but they are limited in scope, not sensitive, and applied inconsistently (Brossette, Hacek et al. 2006). Devices have been cited as sources of infection, both as modes of transmission and reservoirs, but the origin of the pathogens and their transfer to the device are unknown and not widely studied. This raises questions as to whether the devices become contaminated when they are opened, or if their sterile barriers are breached prior to use.

Package integrity is the limiting factor in the maintenance of a product's sterility until use (Schneider 1980) and has been identified as the most important objective for

the medical device industry (Allen 2009). A sterile barrier system (SBS) is defined as the "minimum package that prevents ingress of microorganisms and allows aseptic presentation of the product at the point of use" (ISO 2006; ISO 2006). It is expected, and even assumed, that the contents of a sterile barrier system remain sterile until the package is opened or damaged, leading some to define a "perfect wrapper" as being "impervious to extraneous microbes, liquid-proof, free of holes, free of lint, free of memory, strong enough to resist punctures and tears, and economical to use" (Belkin 2004).

Several factors would have to occur to facilitate the spread of nosocomial infections as a result of failed package integrity. Pathogenic microbes would need to penetrate through or around the sterile barrier, remain viable within the package, and adhere to the device inside the package. Then the device (or other intermediate object which came into contact with the contaminated device, including personnel) would need to transfer the pathogen into the patient's body (Spitz 1994). The problem is that there is no way of knowing if a sterility compromising event has occurred (Webster, Lloyd et al. 2003).

2.6.1 Package Testing

Uncertainty exists as to what size hole is a threat for breaches of sterility. The common belief is that any hole is a threat, no matter what its size is. As a result, integrity tests are becoming increasingly sensitive (finding smaller and smaller holes); however, even if a hole is present, is it possible for a microbe to travel through it, and if so, what are the variables that facilitate this?

The FDA states that products labeled as sterile are expected to be free from viable microbial contamination and that physical tests may be more useful than sterility testing in demonstrating the potential for product contamination (U.S. Department of Health and Human Services 2008). Device manufacturers test their packages to ensure that there are no defects present and sterility of the product is maintained. Most commonly, physical test methods are used, which include strength and integrity tests. Strength tests investigate the strength of seals and resistance to bursting under pressure, while integrity tests are used to identify pinholes or channel defects. Physical tests are commonly used because they are established, economical, and convenient; however, they lack significance because there is no proven correlation between microbial penetration and sterile barrier properties. They identify if defects are present, but not if microbes have penetrated, or can penetrate, though them (Placencia, Arin et al. 1988). They do not specify the defects' sizes or the accuracy of the measuring methods (Axelson, Cavlin et al. 1990). It is necessary to know what the target leak size is that presents a threat before test equipment can be designed and utilized to find them (Floros 1994).

Additionally, consequences exist with integrity testing. Tests that pass packages with defects can impact patient health because there is potential for microbial ingress to occur, resulting in a nosocomial infection. Conversely, tests that fail packages that do not present a risk drive up costs because those products are destroyed unnecessarily (Severin, Bix et al. 2007). Between 1980-84, 31.4% of device recalls were due to holes in

the packaging (Placencia, Arin et al. 1988), but the percentage of those holes that were actually a threat for microbial contamination is unknown.

Integrity tests can be destructive or non-destructive, performed on porous and non-porous barriers, and identify channels and holes. Destructive tests are subjective, typically with pass/fail results, and include bubble and dye testing. Here, companies have to waste product in order to perform the tests. Nondestructive quantitative tests include pressure, force, and vacuum decay methods, which are very sensitive and can detect small leaks down to about 10 µm. Trace gas methods are even more sensitive, detecting leaks a fraction of a micron in size. Visual inspection can only identify channels as small as 75 micron with 60-100% probability and relies on the visual abilities of the inspectors. Other methods include ultrasound/acoustic micro-imaging (Allen 2002), x-ray and thermal imaging (Allen 2003), and helium mass spectrometry (Franks 2003).

Strength tests are also used to assess the mechanical strength of seals and ensure that they are properly bonded so that they will maintain integrity throughout life of the product. These methods include tensile strength and burst or creep testing (Franks 2002).

In 1995 Hansen et al. published a study comparing microbial challenge and physical testing to identify channel defects in film pouch seals. They created defects across the seals using metal shims and wires. Pouches each contained a paper towel and plastic forceps. For the pouches subjected to microbial challenge, a nebulizer was used to aerosolize *Bacilus subtilis* var. *niger* spores (ATCC #9372) with a starting

concentration 1 x 10⁶ cells/ml for 30 minutes within the microbial challenge chamber. Physical tests included dye penetration and visual inspection. To test for microbial ingress, the paper towels and forceps were removed from the pouches after microbial challenge testing and placed in soybean-casein digest broth and observed for growth.

Results indicated that two pouches containing defects, of the 150 pouches that were microbial challenge tested (30 controls and 120 with seal defects), tested positive for the test organism. A false positive was found in one of the control samples and there were four cases of positive findings for organisms other than the test organism. The same packages were also physically tested and the defects were correctly identified in all of the samples, with no false positives for samples without defects.

Researchers concluded that physical tests are more effective than wholepackage microbial challenge testing when evaluating whole-package integrity because of the inconsistency of the microbial challenge method in identifying defects, the positive findings of organisms that were not the test organism, and the false positive in the control pouch (Hansen, Jones et al. 1995).

2.6.2 Factors that Can Affect Microbial Ingress

When performing microbial challenge tests in an effort to understand how sterility is compromised in in-use environments, there are a multitude of variables to consider. These include packaging characteristics, defects, characteristics related to microorganisms, torturous path, methods of microbial challenge, and factors related to transport, storage, and handling.

2.6.2.1 Packaging Characteristics

The type and configuration of packaging materials are important choices for sterile barriers (Webster, Lloyd et al. 2003) and characteristics to consider include geometry, thickness, rigidity and porosity. Rigid trays and flexible pouches are common medical device packages. Trays are typically silicon coated or uncoated polyethylene terephthalate (PET) or glycol-modified polyethylene terephthalate (PETG) (Blocher 2009) that are sealed with porous or nonporous lids. PETG is used for 60% of all preformed medical device trays and is resistant to the hazards associated with radiation sterilization processes (Pilchik 1999). Pouches can be manufactured from a variety of polymers, but a common configuration is a PET/LDPE (low density polyethylene) layer (forming one side) sealed to porous layer (forming the other side). Other materials that are common include Nylon and foil (Blocher 2009).

In 2006, Dunkelberg and Rohmann published a study comparing the penetration of airborne microorganisms into sterile pouches made of transparent plastic film and sterilization paper. The pouches, containing thermoresistant agar dishes, were sterilized, placed in an exposure chamber, and subjected to an aerosol of *Saccharomyces cerevisiae* (baker's yeast) with a concentration of 10⁸ cells/ml. Atmospheric pressure inside the chamber was cycled through 48 pressure changes of 75 mbar. Following treatment, the samples were incubated and inspected for growth.

When agar dishes were placed with their open surfaces under the paper sides of the pouches, 82 of 237 pouches (35%) had contamination. When the dishes were placed under the film sides of the pouches, 3 of 166 pouches (2%) had contamination.

Researchers concluded that it was the paper, not plastic, sides of the pouches that allowed the ingress of microorganisms (Dunkelberg and Rohmann 2006).

2.6.2.2 Packaging Defects

The type and size of defects in the packages are also factors for consideration. Package defects can come in all shapes and sizes and be present anywhere on the package including surfaces and seals. Defect types include pinholes, cracks, sealing failures, and defects in barrier layers (Axelson, Cavlin et al. 1990). Holes or defects in packaging not only increase the risk of microbial contamination and nosocomial infections as a result, but they can also create economic loss due to the costs of treating infections and in the event of product recalls (Chen, Harte et al. 1991). Conversely, microscopic holes may not impair sterile package integrity because a driving force is needed for contamination and a myriad of other factors could affect this. Overly stringent sensitivity levels for package integrity testing may actually reject packages that are effective in maintaining sterility, resulting in economic loss (Jones, Hansen et al. 1995).

Tested defects are commonly pinholes through the sterile barrier or channels within the package seal and their shapes, diameters, and lengths likely affect the ability of microorganisms to travel through them. Allen-Wojtas et al. examined flow rate through microperforations in plastic films. Researchers found that flow rate is dependent on geometric features of both the entry and exit sides of the holes. As area decreases, proportionally more gas molecules are affected by the side walls, which results in a greater than linear reduction in transport rate, essentially increasing

viscosity. Perforations >55 μ m in diameter demonstrated that air convection contributed more strongly to gas transfer. Researchers concluded that small holes are also less likely to transmit contamination and both perimeter and shape may also be factors in transmission (Allan-Wojtas, Forney et al. 2008).

2.6.2.2.1 Creating and Measuring Hole Defects in Packaging

When performing tests to quantify microbial ingress into sterile packaging, it is necessary to produce hole defects that are repeatable and consistent. Producing very small defects (<75 µm) can be difficult (Jones, Hansen et al. 1995). The most common methods include use of orifices or capillaries, mechanical punctures, and laser drilling. When producing holes, both cold and hot needles are slow and only create large perforations (≥1 mm diameter). Hot needles melt the plastic to form holes and then redeposit it as large rims around the hole edges. Cold needles punch rough holes through the film where flaps remain that can cover the holes. Lasers use heat energy to evaporate the plastic (ablation) to produce small, clean holes that are sealed along the edges. Depending on the type of plastic, it may completely evaporate or some may redeposit on the film surface. Microelectric discharge machining (micro-EDM), also referred to as spark machining or spark eroding, is a relatively new technique for microperforations in plastic films (Allan-Wojtas, Forney et al. 2008).

Several researchers have used a variety of methods to create and measure holes in packaging. Keller et al. used nickel microtubes to create seal defects in flexible pouches with internal diameters of 10 and 20 \pm 2 μ m and lengths of 5 and 10 \pm 1 μ m

(Keller, Marcy et al. 1996). Chen et al. used nickel alloy orifices measuring 5, 10, and 15 μ m to create defects in flattop cartons (Chen, Harte et al. 1991).

Maunder et al. created holes in flexible pouches using a No. 14 sewing needle held in hemostatic forceps so that 1.5mm of the needle point was exposed. This resulted in diameters measuring 33-160 μ m, with the longest dimension being recorded for non-round holes (Maunder, Folinazzo et al. 1968).

Hansen et al. created channels across the seals of film pouches using metal shims and wires that were 0.375 to 0.005 inches in diameter. They found that the wire channels could become blocked with adhesive and their average widths were from 0.022 to 0.439 inches. Their method of measurement was done by peeling the package open and measuring the channels at outer edges under 10x and 50x magnification (Hansen, Jones et al. 1995).

Ahvenainen et al. created holes in plastic cups using touch needles with point radii varying down to 2 μ m. The resulting diameters were 5-100 μ m and were measured using 120x magnification and a scale in the ocular. They examined the hole shapes using 198x magnification and determined that touch needles can only be used with very thin packaging material, or the hole will be conically shaped (Ahvenainen, Mattila-Sandholm et al. 1992).

Axelson et al. also created holes in plastic cups using honed sewing needles and touch needles with point radii of about 2 μ m, resulted in diameters of 5-10 μ m. Additionally, they used capillary columns measuring \geq 25 μ m in diameter and drilled holes measuring 300 μ m in diameter. All holes were measured with a microscope.

Axelson et al. also measured holes using electrolytic conductance. This method is based on the theory that the electrolytic conductance of a packaging made of an insulating material is drastically changed by a small hole, provided the hole is filled with a conducting liquid. It also requires knowledge of length of hole and can measure down to 0.8 µm if the holes are not longer than 1 mm (Axelson, Cavlin et al. 1990).

Gilchrist et al. used a laser beam to create holes in trilaminate pouches (PE, aluminum, and PP) that were approximately round with no tears or flaps; they measured 17-81 μ m in diameter. They also punched holes using a stainless steel wire (0.004 inch diameter) that did result in tears and flaps; these measured 22-175 μ m in diameter (Gilchrist, Shah et al. 1989).

Lampi used a laser to create holes in pouch blanks. Preliminary sizing was conducted with a flat field microscope. Then the gas flow rates through the holes were measured, scanning electron microphotographing of defects was conducted, and the hole diameters were calculated. Lampi also created defects by flexing the pouches using an MIT Folding Endurance Tester, which resulted in the smallest hole size of 11 μ m; however, it was determined that flexing was not a likely cause of failure because 1000-5000 flexes were required to create a failure. Additionally, Lampi punctured the pouches with a fine fire-hardened tungsten wire resulting in the smallest hole size of 100 μ m. Lastly, Lampi created holes using abrasion of fold in the pouches against a relatively smooth fiberboard surface. This resulted in the smallest hole size of 24 μ m (Lampi 1981).

Bix et al. created 50µm holes in PETG trays using an excimer laser. The holes were measured using SEM and confocal microscopes. Researchers found that significant differences (p<.0001) existed between the two methods of measurement, with the confocal images having more detail, resulting in larger measurements than those from the SEM. Additionally, researchers found that the entry sides of the holes were larger than the exit sides (p<.0001); the exit sides were closer to 50µm, while the entry sides were 50-100% larger (Bix, Kassarjian et al. 2005).

Laser drilling is an important method for creating very small hole diameters ranging from 10-50 µm, because conventional alternative methods become difficult and cost inefficient. PET is a good candidate for laser drilling because it allows easy observation with optical microscopes. Shorter wavelengths have the best precision for lateral diameters, but sometimes the diameters end up being larger than intended. This is likely the result of liquid film forming on the inner walls during drilling. The liquid becomes accelerated toward the exit side of the hole by momentum transferred from the outcoming gas plume, therefore contributing to an increase in hole diameter (Lazare and Tokarev 2004).

2.6.2.2.2 Microscopy Methods for Measuring Holes

Various microscopic techniques have been employed to measure pinhole defects in packaging. The main differences in microscopy techniques are resolution and depth of focus/field (Allan-Wojtas, Forney et al. 2008). Some methods have been successful in capturing images of the entry and exit sides of the holes on the surfaces of the packaging materials; however, differences have been found between measurements of

the same holes using different types of microscopes. Additionally, differences in hole diameters were found between the entry and exit sides of the holes (Bix, Kassarjian et al. 2005). This raises questions as to what shape and size is within the entire hole tunnel, in between what the microscopes can image at the surfaces.

Ghosh and Anantheswaran used a Ziess Photoscope II to measure microperforations in film that were created with mechanical sparks. Researchers printed the magnified images and measured hole diameters in four places 45 degrees from one another and then averaged the values. A photograph of a 0.1 mm stage micrometer was used for calibration. The perforations (which included film deposits around the edges of the holes) were sliced through with a razor blade in preparation for thickness measurements. Then the samples were coated with gold/palladium in a BAL-TEC SCD050 sputter coater and SEM images were obtained with a 10kV JOEL 5400 SEM. Images were recorded on Polapan 100 film and transferred to a Princeton-Gamma Tech Integrated Micro-analyzer for further imaging and x-ray to generate thickness measurements. The cut edges were digitized to generate measurements in microns. Five measurements were taken from random locations within perforations and their averages were calculated. Their resulting measurements ranged from 96-247 μm in diameter and 50.7-76.9 µm in thickness. Additionally, perforation thickness increased as diameters increased, due to larger amounts of the film being deposited around the microperforation edges when the holes were created (Ghosh and Anantheswaran 2001).

Piergiovanni et al. measured hot needle perforations in film using two imaging techniques. First, researchers placed the film a on scanner, covered it with a mirror,

collected digitalized grey scale images with 1200 pixel/in² resolution, and stored them in TIF format to determine the density of the holes per cm². Second, Dia-Brightfield microscopy, with a computerized system for image processing and analysis, was used to collect images for diameter measurements. Here, four measurements were also taken 45 degrees apart from one another and the averages were calculated; this was done for both the internal and external sides of film. While no statistically significant differences were identified between sides, the internal sides had more defined borders while the external sides had uneven borders. Polarized light was then used to identify irregular crests of melted polymer coming out from the horizontal planes. Researchers stated that the presence of crests could reduce entry of contaminants through the holes. The film was also subjected to artificial sweat contamination with a compressive machine and cotton pads containing sweat simulant, and artificial sneezes with a manual aerosol dispenser containing saliva simulant. Researchers concluded that the risk of contamination was highly correlated to hole surface area and it was likely that artificial saliva and sweat transmission through perforations is proportional to the hole dimensions and densities (Piergiovanni, Limbo et al. 2003).

Allan-Wojtas et al. compared three microscopy methods (light, SEM, LV-SEM) to measure microperforations in 1 mil polyethylene and polypropylene films. With light microscopy, photons illuminate the sample and that interaction provides microstructural information. Brightfield transmitted light microscopy (BFTLM) requires minimal sample preparation and is best for thin samples that have some inherent contrast or can be stained. Differential interference constrast light microscopy (DICLM)

can be used to observe local differences in thickness for samples with little contrast because the edges between two areas with different refractive indices are enhanced. The light microscopy techniques used included observing the upper and lower surfaces of the films with 40x magnification under Brightfield conditions and then switching to DICLM conditions using a Nikon Eclipse E800 light microscope equipped with a Nikon DXM 1200 digital camera. Grayscale images were captured as digital files with 3600 x 2880 pixels (Allan-Wojtas, Forney et al. 2008).

Scanning electron microscopes (SEM) work by scanning the samples with an electron beam and creating images from that interaction. SEM works well for thicker samples because light clarity is better than with light microscopy. The SEM samples were coated with gold/palladium using a Hummer VII sputter coater and observed with a JEOL T330A SEM operated at 5kV. Images were recorded on Polaroid Type 55 positive/negative film, and the negatives were scanned into grayscale digital files (1320 x 1056 pixels) using a Polaroid SpringScan45 Ultra scanner (Allan-Wojtas, Forney et al. 2008).

Low-vacuum SEM (LV-SEM) was conducted using a Quanta 200 environmental SEM (ESEM) under low-vacuum mode (20kV, 133.0 Pa). Both sides of the samples were observed and images were captured as grayscale digital files (1024 x 884 pixels) (Allan-Wojtas, Forney et al. 2008).

These methods were used to determine the area, perimeters, and diameters of the microperforations. Area was defined as the region bound by the plastic rim and perimeter values were measured from the inside edges of the plastic rims. Hole shape

was also observed to determine roundness using an estimate of circularity (Allan-Wojtas, Forney et al. 2008).

Accurate measurements were not possible with Brightfield microscopy because distinct hole edges were not visible; they were only in focus at the upper and lower surfaces of film. Additionally, both sides of the holes varied in structure and size, indicating the hole tunnels were not cylindrical (Allan-Wojtas, Forney et al. 2008).

DICLM was able to provided visible areas of contrast between the holes and their rims, such that the edges and deposits of film around them were observed. This method showed that the rims were thicker than the surrounding plastic and was determined to be suitable for quick measurements (Allan-Wojtas, Forney et al. 2008).

The SEM showed distinct structural and topographical characteristics that were clearly visible due to the increased depth of focus. This method was suitable for more accurate diameter measurements because the hole edges were well defined (Allan-Wojtas, Forney et al. 2008).

While the LV-SEM offered same depth of focus as the SEM, it did not require altering the samples to prepare them for imaging. This allowed the samples to be used for other tests and they could also be quickly removed and remounted so that the other sides could be observed. Researchers concluded that this was the best technique for studying microperforations (Allan-Wojtas, Forney et al. 2008).

2.6.2.3 Torturous Path and Layers of Packaging

Torturous path is the physical difficulty of the "route" through the package that a microorganism needs to travel in order to breach the sterile barrier. This can include

the microscopic routes through defects and material surfaces as well as relatively larger routes across or around multiple layers of materials.

Discrepancies exist on the number of layers that a package needs to ensure sterility. A single layer of packaging requires less labor and costs less, but it is generally believed that a double layer is essential for the practice of asepsis. A survey of nurses indicates that they prefer double layers over single layers because it allows them easier aseptic presentation of the devices into the sterile field (Blocher, Neid et al. 2010). The outer layer is intended to protect against dust particles that could contaminate the contents when the package is opened and is supposed to be removed before the pack is delivered to the clean zone (Belkin 2004). It has also been suggested that safe storage time is significantly increased with multiple wraps because an increased number of layers will provide a protective barrier that creates a torturous path for microorganisms to travel through as well as prevent tears and punctures (Spitzley 2002). But, "if something can be wrapped once instead of twice, provide just as good a barrier, and be delivered to the field without compromising sterility, why take the time and expense to wrap it twice?" (McCormack 1995).

At least one study suggests that multiple layers of packaging may actually inhibit aseptic presentation. In 2008, Crick et al. published a study comparing the opening of multiple, individually double-wrapped, orthopedic implant screws vs. screw banks (one package containing multiple screws). Five nurses each bathed their hands in Glitterbug (a cream that is visible under ultraviolet light) and opened 20 double wrapped screws and one screw bank. Of the 100 double wrapped samples, one had contamination.

None of the screw banks had contamination. They concluded that opening individually wrapped items increased the risk for potential contamination of an operative field as compared to opening a single package and recommended further investigating a move towards minimizing the number of separately wrapped sterile packages during a surgery (Crick, Chua et al. 2008).

This idea runs counter to early research, which suggests that multiple layers of packaging are necessary to increase storage time. In 1973, Standard et al. published a study examining microbial penetration of sterile packs with multiple layers. The three configurations were described as: double-wrap (two layers each) muslin, single-wrap (two layers) muslin inner covering with single-wrap (one layer) two-way crepe paper outer covering, and single-wrap (two layers) muslin inner covering with single-layer BAR-BAC wrappers. These were used to wrap gauze sponges and stored on open shelves in a central sterile supply department in a hospital. Packs were chosen in groups of two or four at weekly intervals and assayed for growth.

The packs with double-wrap muslin had microbial penetration at 28 days. The single-wrap (two layers) muslin inner covering with single-wrap (one layer) two-way crepe paper outer covering had penetration at 77 days. The single-wrap (two layers) muslin inner covering with single-layer BAR-BAC wrappers had penetration at 63 days. With the objective of increasing storage time before sterility is compromised, these results led to recommendations that include using sterile impervious plastic bags, closed-cabinet storage, and more than single layers of packaging (Standard, Mallison et al. 1973).

Other research further supports the design of systems composed of multiple layers. In 2004, Dunkelberg and Wedekind published a study examining contamination of sterile packages with multiple layers. The packages were described as wire baskets with sterilization sheets affixed using adhesive tape. There were four configurations: single-paper packaging (P), double-paper packaging (PP), textile and paper double packaging (TP), and double packaging with transport packaging (TPP). They were subjected to repetitive mechanical stress by being pressed approximately 1 to 2 cm five times per minute with a 1 kg weight for 0, 1, and 3 hours. This was conducted in two separate rooms with mean ambient airborne bacterial counts of 35 and 440 CFU/m³. Following the mechanical stress, the packages were incubated and inspected for growth. Results indicated that mechanical stress led to time-dependent contamination and that barrier efficacy increased with the number of layers of packaging (Dunkelberg and Wedekind 2004).

In 2006, Dunkelberg published another study with Rohmann comparing the performance of single- and double-layer pouches composed of transparent plastic film and sterilization paper when atmospheric pressure changes were the driving force. The pouches were sterilized containing thermoresistant agar dishes, placed into an exposure chamber, and subjected to an aerosol of *Saccharomyces cerevisiae* (baker's yeast) with a concentration of 10⁸ cells/ml. Twenty-five samples of each were exposed to 4 hours in the exposure chamber with 12 atmospheric pressure changes of 75 mbars occurring per hour. Following treatment, they were incubated and inspected for growth. Four percent of the single-layer pouches had contamination while none of the double-layer

pouches did. They concluded that double-layer pouches will provide a sufficient sterility assurance level in clinical settings (Dunkelberg and Rohmann 2006).

These studies examine the use of multiple layers of packaging in environments which require aseptic presentation and in storage and transport environments. The study performed by Crick et al. suggests that the more times that devices are opened, the greater the probability for a contaminating event to occur. The studies performed by Standard et al, Dunkelberg, Wedekind, and Rohmann suggest that the greater the number of layers, the longer the devices will remain sterile in transport or storage environments. This suggests that more layers of packaging will maintain the devices' sterility up to the point of use, but it also suggests that increased layers of packaging at the point of use increase the probability for a patient to acquire an infection.

2.6.2.4 Microorganisms

Characteristics related to microorganisms can also affect microbial ingress of sterile packaging. Factors to consider include type, size, mode of motility, concentration, the duration of exposure, and viability.

2.6.2.4.1 Nosocomial Microorganisms and Pathogenicity

Nosocomial infections can be bacterial, viral, or fungal. Bacterial infections are those that are typically reported and are also becoming more worrisome due to the rapid increase of drug resistance in these organisms. While there are seven classes of antibiotics for treatment, there is resistance to at least one antibiotic in at least 70% of HAIs (Kowalski). The most common bacterial infections in humans tend to be those that are gaining antimicrobial resistance. Nosocomial infections are among these and

examples include: penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant *enterococci*, methicillin-resistant *Staphylococcus aureus* (World Health Organization 2002).

Viral infections also represent a significant fraction of HAIs and, like their bacterial counterparts, have the potential to evolve rapidly, due to high mutation rates. There are less vaccines available than there are viruses to treat because each variant of a virus requires a specific antiviral agent and vaccine. Viral infections are also worrisome because epidemics can occur before vaccines are developed (Kowalski 2007).

Fungal infections, while on the rise, are not typically considered nosocomial. They are associated with immunodeficiency diseases that can facilitate their spread. Like viruses, they have a small number of fungicidal agents that can be used for treatment (Kowalski 2007).

The number of viable virulent microorganisms required for a 50% probability of infecting a patient, also referred to as minimum infectivity number (MIN), depends on the virulence of the microorganism, site of entry into the patient, health status of the patient, and the normal route of infection upon entry (Allison 1999). Malone and Larsen suggest that the microorganisms most commonly linked with nosocomial infections are Enterococci, *E. coli, Pseudomonas sp., S. aureus,* and *Candida sp.* (Malone and Larson 1996). Richards suggests the most common pathogens and their corresponding infections are: *S. aureus* for nosocomial pneumonia with a rate of 17%, coagulase-negative *staphylococci* for BSIs with a rate 39%, *E. coli* for UTIs with a rate of 19%, and *Enterococcus* for SSIs with a rate of 17% (Richards, Edwards et al. 2000). Allison

suggests that *Pseudomonas aeruginosa* is of greatest concern for producers of sterile products, particularly those for eyes (Allison 1999).

2.6.2.4.2 Test Microorganisms and Microbial Concentration

When choosing a test organism, not only does it need to be an accurate model for a nosocomial pathogen, but its concentration and safety for use also need to be considered. Like the other factors that are known to impact penetration rates, researchers have used a variety of microorganisms and concentrations for package integrity testing.

In 1996, Keller et al. published a study examining the effects of microorganism motility and microbial concentration on ingress through microtubes in flexible pouches. They compared motile and nonmotile *Pseudomonas fragi* at concentrations of 10² and 10⁶ CFU/ml. Six of 127 pouches tested positive for contamination. All of these were exposed to 10⁶ CFU/ml. They concluded that contamination was affected by both level of motility and concentration of the test organism (Keller, Marcy et al. 1996).

Chen et al. used *Lactobacillus cellobiosus* at a 2.5 x 10^6 cells/ml concentration (Chen, Harte et al. 1991). Hansen et al. used *Bacilus subtilis* var. *niger* spores at a 1 x 10^6 cells/ml concentration (Hansen, Jones et al. 1995). Dunkelberg and Rohmann used *Saccharomyces cerevisiae* (baker's yeast, which is approximately 10 µm in diameter) (Dunkelberg and Rohmann 2006). Dunkelberg and Schmelz used *Micrococcus luteus* at a 10^8 cells/ml (Dunkelberg and Schmelz 2009).

2.6.2.4.3 Viability on Packaging

In addition to the questions surrounding the ability of microbes to breach the sterile barrier of a package, there has also been uncertainty regarding whether or not microbes will remain viable inside the package until the time of use. Evidence suggests that their viability is a realistic concern, especially with one of the most threatening bacteria in existance, MRSA.

In 2001, Dietze et al. published a study on the survival of MRSA on sterile goods packaging. The packaging materials they used were single-use syringe wrappers composed of a paper side and laminated side consisting of foil, polyethylene, and polyamide. The samples were contaminated with a MRSA suspension by micropipette (0.1 mL on the paper sides and 0.05 mL on the laminated sides) and stored in conditions representative of a storage closet without light, dust-protected, and at 20°C and 20-30% RH. At regular intervals samples were inspected and researchers found that the MRSA survival time was greater than 38 weeks. At 50 weeks, there was no further survival. Paper and foil had similar survival rates (Dietze, Rath et al. 2001).

2.6.2.5 Methods of Microbial Challenge

Microorganisms that may contaminate packages are present in the atmosphere as both dust particles and water droplets (Floros 1994). When conducting microbial challenge testing on packaging, there are a few representative options to choose from; these typically include aerosols, submersions, or talc methods.

Literature indicates that aerosol methods are most commonly used for microbial challenge of packaging. Understanding how aerosolized particles containing microbes

travel through microscopic defects in the packages is intended to be used to set realistic limits on the critical leak size for package integrity (Floros 1994). Aerosols consist of small particles suspended in a gaseous medium and the type of microorganism, concentration and viscosity of the microbial suspension, its contact time with the test packages, and pressure differences are all factors for consideration (Gnanasekharan and Floros 1995).

The microbial challenge systems previous researchers have used generally incorporated some form of chamber with a system for aerosolizing microbial solutions. Keller et al. utilized a Plexiglass[®] chamber with two aerosol entry ports. The aerosol was created using nebulizer kits (Keller, Marcy et al. 1996). Chen et al. also used a Plexiglass[®] chamber. This had a recirculation system with two pumps that forced fluid through 32 nozzles mounted on the all sides of the chamber (Chen, Harte et al. 1991).

2.6.2.6 Transport, Storage, and Handling

It is generally believed (and somewhat intuitive) that the probability of a sterility compromising event occurring increases with both time and handling (Webster, Lloyd et al. 2003) and that shelf life depends not only on the quality of the material, but also on transport, storage, and handling conditions (Belkin 2004). Packages can be subjected to risk during contact with atmospheres in processing, storage, distribution, and in-use environments (Gnanasekharan and Floros 1995) and one of the factors of concern here are pressure differentials across the sterile barrier.

Transportation conditions, such as shock, vibration, and compression, are routinely simulated to test packages in laboratory settings; however, it is difficult to

simulate handling conditions. It is unknown how package handling within the hospital environment affects sterility, such as when packages are put into bins or nurses' pockets (Allen 2010). Additionally, it is also unknown how the number of times a package is handled before its final use affects sterility (Webster, Lloyd et al. 2003). Factors that could impact this include conditions within the hospital environment, such as the concentration of bacteria in ambient air (Dunkelberg and Wedekind 2004), and static charges (Hansen, Jones et al. 1995).

The type of storage areas and their conditions may also have an effect on microbial ingress and shelf life. Storage shelving can be open or closed cabinet (Webster, Lloyd et al. 2003), and researchers suggest closed cabinet storage will offer better protection for sterile packages (Standard, Mallison et al. 1973). The conditions of the storage area are also relevant and include levels of cleanliness, temperature, and humidity (Webster, Lloyd et al. 2003). Again, multiple layers of packaging may also produce longer sterile storage, such as with device packs that are sealed into sterile impervious plastic bags (Standard, Mallison et al. 1973).

Another factor that can compromise sterility is pressure differential across the sterile barrier. This serves as a driving force to pull microorganisms through microscopic defects in the packaging. Pressure differences can occur during transportation by aircraft or in vehicles traveling over mountain passes. They can also occur within the hospital through general handling, when a package is transferred to an area with positive pressure such as an operating room with hyperbaric pressure, from transport within an elevator (Dunkelberg and Fleitmann-Glende 2006), by opening and closing of
storage closets and drawers (Allen 2010), and from pressure pulses created by heating and cooling systems (Spitz 1994). Weather changes can also create pressure differentials (Dunkelberg and Wedekind 2004).

2.6.2.6.1 Pressure Differential

The flow of gas may be the most serious threat to sterility of a packaged device (Hackett 2001). Microorganisms can be carried by air currents into packages or fall through defects due to gravity (Hackett, Scholla et al. 2000). Flow is dependent on pressure differential, headspace within the package, and time (Spitz 1994). When a pressure differential is induced on a package, it forces headspace gas to flow out or surrounding gas to flow in. This can change the geometry of the package, including shape, volume, and rigidity, and the headspace and surrounding gas pressures (Gnanasekharan and Floros 1995).

A major cause of pressure differential during transport is the ascent and descent of aircrafts (Hackett 2001). Airborne microorganisms moving at very low velocities, low pressure differentials induced from handling or air and ground transportation, can be a threat for sterile packages (Hackett, Scholla et al. 2000). A pressure differential of -3.78 psi simulates and aircraft descent from 8,000 ft (Severin 2006) and researchers suggest that approximately 3 lb of pressure is required to overcome capillarity in a 10 μ m diameter hole; therefore, a 3-4 psi pressure differential is reasonable for microbial challenge testing (Lampi 1981).

2.6.2.6.1.1 Pressure Differential Research

Researchers have studied the effects of pressure differential on microbial ingress of various sterile packages. Studies include the effects of pressure levels, the number of pressure changes, combined effects of pressure and microbial concentration, and simulated transport environments.

In 2006, Dunkelberg and Fleitmann-Glende published a study examining pressure differential on the microbial barrier effectiveness of reusable sterilization containers with filters incorporated in the lids in an effort to prevent nosocomial infections. They tested 105 standard containers (58 x 28 x 15 cm) and 111 half-size containers (29 x 28 x 25 cm) that were furnished with paper, textile, or permanent plastic filters. Thermoresistant agar plates (15 x 20 cm) were placed into the containers prior to sterilization. Test conditions included two hours in an exposure chamber with an aerosol of baker's yeast suspension (concentration = 10^8 cells/ml). There were 12 atmospheric pressure changes per hour that reduced and increased pressure by 1000 Pa with a maximum level of 7000 Pa. Upon completion of testing, the containers were incubated and inspected for growth.

Of the standard containers, 9 out of 11 with paper filters, 70 out of 79 with textile filters, and 1 out of 15 with permanent plastic filters had growth on the agar plates. Of the half-size containers, 9 out of 11 with paper filters, 70 out of 79 with textile filters, and 0 out of 15 with permanent plastic filters had growth. Their recommendation was to use quantitative microbial challenge testing with pressures between 25 and 70 hPa at one-year intervals to identify containers that are no longer

effective barriers in an effort to prevent nosocomial infections (Dunkelberg and Fleitmann-Glende 2006).

2.6.2.6.1.2 Number of Pressure Changes

In 2006, Dunkelberg and Rohmann published a study examining how the number of pressure changes affected the contamination of pouches. Researchers developed a microbial challenge test to detect penetration of airborne microorganisms into sterile pouches comprised of transparent plastic film and sterilization paper. The pouches were sterilized containing thermoresistant agar dishes, placed into an exposure chamber, and subjected to an aerosol of *Saccharomyces cerevisiae* (baker's yeast) with a concentration of 10⁸ cells/ml. Atmospheric pressure inside the chamber was periodically changed to simulate weather-dependent pressure changes. Following treatment, samples were incubated and inspected for growth.

Researchers found that contamination of the pouches was dependent on the number of decreases in atmospheric pressure. When pouches were subjected to periodic pressure changes in increments of 25 mbar cycling between 0 and 75 mbar (each cycle lasting 4 to 5 minutes), the number of contaminated pouches increased with the number of pressure changes. One hundred pouches were used for each test group. The numbers of pressure changes used for each group of 100 were 0, 6, 12, 24, 36, and 48 with contamination occurring in 0, 1, 2, 8, 9, and 11% of the pouches (Figure 8).



Figure 8. Effect of the number of pressure changes on the percentage of contaminated pouches (Dunkelberg and Rohmann 2006).

2.6.2.6.1.3 Pressure Differential and Microbial Concentration

Dunkelberg and Rohmann also examined the combined effects of pressure with microbial load. When comparing two microbial aerosol concentrations (10⁷ and 10⁸ cells/ml) in the presence of simulated atmospheric pressure changes, pouches subjected to the higher concentration had greater contamination compared to those subjected to the lower concentration (Dunkelberg and Rohmann 2006).

2.6.2.6.1.4 Pressure Differentials Simulating Transport and Storage

In 2009, Dunkelberg and Schmelz published a study on the efficacy of sterile barrier systems against microbial challenge under pressure differentials simulating those in transport and storage. Here, they combined levels of pressure and the number of pressure changes as test factors. Two styles of flexible peel pouches were tested: paper with plastic film material and nonwoven (HDPE fibers) with plastic film material. Each pouch contained an open agar plate so that one test series had the open sides under the paper or nonwoven surfaces and another test series had the plates under the plastic film surfaces. The pouches were subjected to 20 periodic atmospheric pressure changes of 50 and 70 hPa in a microbial exposure chamber; each cycle period lasted six minutes for a total of two hours. A nebulizer was used to aerosolize *Micrococcus luteus* at a 10⁸ cells/ml concentration within the chamber.

Growth was only observed when the plates were under the paper or nonwoven sides of the pouches. For the paper pouches, there was contamination in 30 of 99 plates (30%) and 48 of 100 (48%) plates after the 50 and 70 hPa exposures, respectively. For the nonwoven pouches there was contamination in 3 of 99 (3%) plates and 7 of 99 (7%) plates after the 50 and 70 hPa exposures, respectively (Figure 9).



Figure 9. Effects of pressure differentials for paper and nonwoven material rates of contamination (Dunkelberg and Schmelz 2009).

2.6.3 Investigation of Microbial Ingress through the Sterile Barrier

HAIs are a significant cause of morbidity and mortality in the United States that drive up the cost of healthcare. Despite the practices employed in infection control programs, they are still occurring and have not been eliminated. They are also becoming more difficult to treat due to increased drug resistance, which adds urgency to the situation. While multiple causes of HAIs have been identified, it is difficult to trace infections back to a source. When infections are linked to a device, it is still unknown how the device became contaminated.

Packaging and the sterile barrier have been largely overlooked as a source of infection. Healthcare workers appear to trust that the devices they use are sterile at the point of use and do not suspect breeches of the sterile barrier. Device manufacturers are diligent in their efforts to ensure sterility; however, there are limitations with the test methods currently available. Even when a defect can be found in a package, it is unknown whether it can be penetrated by microorganisms. A limited amount of research studying factors that affect microbial ingress has been published and the results, although suggestive, are inconclusive. Variables that have been studied include hole size, pressure differential across the sterile barrier, and the effects of multiple layers of packaging.

The purpose of this research is to study the combined effects of these three variables in realistic packaging configurations in an effort to identify how they affect microbial ingress across the sterile barrier. Understanding what facilitates the ability of microbes to traverse the sterile barrier can be applied toward tracing sources of

infection and improving package/device design to maintain the sterile barrier and aid in aseptic technique.

PART 1: EFFECT OF PRESSURE DIFFERENTIAL ON MICROBIAL INGRESS OF STERILE MEDICAL DEVICE TRAYS WITH 100 μm PINHOLES

3.1 Objectives

Part 1 of this study builds on the work of Severin (2006) entitled "The Effect of Pressure Differential on Microbial Penetration of a Sterile Medical Device Tray". Severin developed a new microbial challenge methodology and examined the effect of pressure differential on microbial ingress into medical device trays through varying pinhole sizes. Reported findings indicated that one tray with a 100 µm pinhole out of eight tested had ingress in the absence of an induced pressure differential. These results suggested that further investigation was necessary and the following research objectives were developed:

- to further validate that the microbial challenge methodology developed by Severin (2006) does not produce false positives or negatives.
- to confirm with a larger sample size that microbial penetration can occur in trays with 100 µm pinholes in the absence of pressure differential.

3.2 Materials and Methods

The methods employed to investigate all objectives included the following steps:

- aseptically filling sealed, sterile device packages with a known volume of appropriate sterile growth medium,
- 2. exposing the packages to an aerosolized microbial challenge,

- inducing pressure differential on the packages inside the microbial challenge chamber,
- 4. incubating the packages,
- 5. inspecting for growth,
- and recording binary (growth or no growth) and variable (number of colony forming units) data.

A total of thirty-two samples were tested to investigate the study objectives. Each contained a 100 μ m pinhole and was sealed with a nonporous lid. Of these, two samples were damaged during testing and removed from the study. All samples were uniquely identified and aseptically injected with sterile growth medium.

The samples were subjected to an aerosolized microbial challenge with a solution of *Escherichia coli* K12 ATCC Number 29181 that had a starting concentration of 1×10^{6} cells/ml. The aerosol was sprayed for 15 seconds and allowed to settle for 30 minutes before the samples were removed from the exposure chamber. Immediately following aerosolization, a pressure differential was induced at random positions within the chamber on two of the test samples per run by withdrawing a known volume of air from the headspace of the tray with a needle and syringe (Severin 2006). When all testing was completed, of the usable trays, 15 had a pressure differential induced across the sterile barrier and 15 did not. The 32 samples were exposed to the test conditions in eight runs of four trays each. Run number and sample position within the chamber were recorded.

Independent variables for model consideration included: sealing run, sealing position, injection run, injection frame position, chamber run, chamber position, and the presence or absence of pressure differential.

3.2.1 Holes

The pinholes in the test trays were thermal laser drilled (Lenox Laser, Glen Arm, MD) with nominal values of 10 μ m (+/- 10%) or 100 μ m (+/- 10%) in diameter; test trays that contained holes each had one hole drilled in the bottom of the tray. The holes were flow calibrated and each tray was labeled with the tray ID, flow rate, and hole diameter. (See Appendix A for a complete itemized materials list.)

3.2.2 Tray Sealing

The test trays used (Figure 10) were "Medtronic Inc. Outer Tray Part No. 350215-001" 0.025 inch (preform thickness) blue tint uncoated polyethylene terephthalate (PETG) trays (Perfecseal, Mankato, MN).



Figure 10. Test trays. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis (or dissertation).

Trays were sealed with LKF-002 Paper/PE/Foil/PE/HSC die cut lids (Amcor Flexibles Healthcare, Madison, WI) (Figure 11) using a CeraTek model MD-2420 shuttlestyle heat sealer (SenCorp, Hyannis, MA) fixtured with a Teflon impregnated fiber glass barrier blanket. There were nine sealing positions that were recorded for each tray per run (Figure 12).



Figure 11. Tray lids.

Figure 12. Sealing positions.

Sealing parameters were: 300°F, 70 psi, 2.5 seconds of dwell time. After sealing, trays were visually inspected for defects according to ASTM F 1886 Standard Test Method for Determining Integrity of Seals for Medical Packaging by Visual Inspection (ASTM 2004); those with defects were removed from the study. Trays were packed inside a double-layer of PE bags, twist tied shut, stacked into corrugated shippers, and shipped via UPS to a sterilization facility for gamma irradiation.

3.2.3 Sterilization

Gamma sterilization was provided by Smith & Nephew, Inc. (Memphis, TN) and performed by Sterigenics (West Memphis, AR) according to process procedure P000257 Rev. M.

3.2.4 Non-destructive Leak Testing

All lidded trays were subjected to non-destructive leak testing. This was done in addition to visual inspection to verify that the trays did not contain defects (other than the intended pinholes) across their sterile barriers. (See Appendix B for results tables.)

Testing was done using an ATC, Inc. Model VE2 supported with the Leak-Tek© data acquisition program (Indianapolis, IN) (Figure 13). The unit functions based on mass conservation law; once the test specimen is pressurized and reaches steady state pressure, the amount of mass flow into the specimen is equal to the amount mass flow that leaks out. Micro-flow sensors, or Intelligent Gas Leak Sensors (IGLS), measure the mass flow extracted from the test specimen to maintain a steady vacuum condition.



Figure 13. ATC, Inc. Model VE2 non-destructive leak tester.

3.2.5 Agar Injection of Lidded Trays

Glass bottles that each contained approximately 30 ml of sterile nutrient growth agar (enough for a single test sample) were prepared by the Media Prep Lab at Michigan State University (East Lansing, MI). The bottles were sealed with foam stoppers and autoclave tape was placed over them to verify the autoclave process was completed (Figure 14). Once the autoclaving was finished, the autoclave bin containing the bottles was transferred into an insulated box for transport. The box was designed to keep the bottles hot so that the agar did not harden before they could be placed in a water bath at the location for agar injection.



Figure 14. Glass bottles containing sterile nutrient growth agar.

Prior to working in the lab, all table tops were disinfected using Multiterge (5 ml in 100 ml of water). The water bath that contained the agar-filled bottles was turned on one hour prior to agar injection. The control and safety thermostats were set between 53-60°C. The water bath was filled to a level slightly above the agar level inside the

bottles. If the water level is too low, the agar can begin to solidify; if it is excessively higher than the agar level, condensation can form on the interior walls of the bottles.

Sterile trays that were injected with sterile agar were each subjected to the following process: The tray was visually inspected a second time for any defects; trays with defects were removed from the study. A 2 in² section in the center of the lid was disinfected for approximately 30 seconds using a 70% isopropyl alcohol swab (Figure 15). Two pairs of forceps were dipped in 70% isopropyl alcohol and flashed with a Bunsen burner. A pre-cut piece of self-sealing septum (Mocon, Minneapolis, MN), approximately 2 cm² in size, was handled as follows: The backing was removed from the adhesive side. The septum was dipped into 70% isopropyl alcohol and flashed with a Bunsen burner (Figure 15). The septum was placed onto the previously disinfected surface of the tray lid and pressed firmly with the forceps so that it fully adhered (Figure 15).



Figure 15. Microbial challenge methodology (Severin 2006). (Reprinted with permission.)

The tray was then placed in the injection frame and position was recorded; the frame had four positions. Injection run was also recorded. Trays were oriented with the lid-sides down so that trays containing holes had the hole-side at the top to prevent the holes from becoming blocked with agar (Figures 16 and 17).



Figure 16. Figure 17. Injection frame with test samples: top view (Figure 16), bottom view (Figure 17).

Next, the septum on the tray lid was disinfected for approximately 30 seconds using a 70% isopropyl alcohol swab (Figure 15); care was used to ensure that only the septum, not any of the lid surface, was swabbed because the septum would come loose if the lid got wet.

An agar bottle was removed from the water bath; one bottle containing 30 ml of agar was used for each tray. The autoclave tape was carefully pulled off of the foam stopper so that the stopper remained intact. The stopper was disinfected for approximately 30 seconds using a 70% isopropyl alcohol swab. Both a 60 ml syringe (Becton, Dickinson and Company, Franklin Lakes, NJ) and an 18G1½ needle (Becton Dickinson and Company, Franklin Lakes, NJ) were aseptically removed from their packages so that the needle could be placed on the syringe; a single syringe was used for each tray. The needle was gently pushed through the foam stopper (using care to not push the stopper into the bottle) and the bottle was tipped onto its side so that the agar could be drawn into the syringe. Once complete, any air in the syringe was pushed out, and the first needle was removed. Then a 16G1 vented needle (Becton, Dickinson and Company, Franklin Lakes, NJ) was opened placed on the syringe so that it could be used to inject the agar into the sterile tray. The use of the vented needle allowed for out gassing from the non-porous package as the agar was injected. The needle was pushed through the septum on the lid of the tray (oriented so that the lid was on the bottom) (Figure 17) at an angle so that it did not pierce through the tray (on the top) (Figure 16) as the agar was injected. The frame was tipped and held forward so that when the agar was injected, it did not surround the injection site. The agar was slowly pushed out of the syringe into the tray so that it covered the lid surface on the bottom and did not touch any of the other surfaces inside the tray. Once the entire volume of agar was injected, the syringe was removed from the tray and the frame was tipped back to its resting, flat position. The tray was gently agitated by rocking the frame so that the agar covered the lid surface evenly. Once a set of four trays was complete, they were removed from the frame and set aside.

Once all of the trays had undergone agar injection, the septums for pressure differential were placed on them. This was done using the same procedure as for the placement of the agar injection septums. The septums were centered on the front sides of the trays.

If any errors occurred during this procedure (inadvertent puncture, etc.), those samples were removed from the study. All samples were stacked in corrugated boxes and held at room temperature until the following day when microbial challenge testing was conducted.

3.2.6 Preparation of Dilution Series Agar Plates

To prepare sterile nutrient growth agar, Difco[™] Nutrient Agar powder was mixed with deionized water. As a benchmark, 23 g of powder can be mixed with 1000 ml of deionized water to make a batch of agar.

This procedure was used to prepare six dilution series agar plates. Each plate requires approximately 15 ml of agar; therefore, 6 plates x 15 ml = 90 ml of agar. Since the amount of agar per plate does not need to be exact, and to simplify the measurements for the procedure, agar was prepared in 100 ml batches.

A 500 ml Erlenmeyer flask was filled with 100 ml of deionized water. (It is important that the depth of the liquid does not exceed roughly 1.5 inches to ensure that the autoclaving conditions reach the center). Using a spatula to transfer the powder into a boat, 2.3 g of powder was weighed on scale. The powder was then poured into the flask. The flask was gently swirled to dissolve the powder. The opening of the flask was sealed with aluminum foil and a piece of autoclave tape was placed on top of the foil.

The flask was put into an autoclave bin, the bin was placed inside an autoclave, and the door was closed. The cycle time was set at 25 Sterilize minutes (Dry time = 0). The orange Fluid Cycle button and then the red "On" button were pressed. The autoclave pressurized to 15 psi and the cycle began when the green and red arrows align at 121°C.

Once the 25 minute cycle was completed, there was an additional wait of 10-15 minutes to allow the pressure and heat to come down from the cycle. Then the door

was slowly opened (to allow for careful steam release). The bin was then removed using thermally insulated gloves.

To pour the agar into the petri plates, six plates were stacked on top of one another. The foil was removed from the top of the flask using care to not contaminate the sterile opening. Then the lid of the top plates was removed and held with one hand (maintaining the lid's orientation as it was lifted) while the other hand poured approximately 15 ml of agar (enough to coat the bottom) into the plate. The lid was then replaced and the plate was lifted from the pile and set aside. During this movement, it was important that the flask was continuously held at the pour angle to prevent drips from going back into the flask and contaminating the sterile agar. This process was continued until all six plates were filled with agar. The plates were kept at room temperature to solidify until use the following day.

3.2.7 Microbial Solution Preparation

The microbial solution used Butterfield's Solution as a diluent for the test organism, *Escherichia coli* K12 ATCC Number 29181 (Figure 18).



Figure 18. Escherichia coli K12 ATCC Number 29181 (Severin 2006).

To prepare the Butterfield's Solution, a 2L bucket was filled with 1L of distilled water. Using a spatula to transfer 68 g of Potassium Phosphate, Monobasic, Crystal (KH₂PO₄) to a boat, the KH₂PO₄ was weighed on a scale and then poured into the bucket. A stir bar was added to the bucket and a Corning Stirrer was used to dissolve the KH₂PO₄. A pH meter was placed in the solution. Wearing latex gloves, 1N Sodium Hydroxide (1N NaOH), was added to the solution with a pipette until the pH was brought up to 7. A pH strip was dipped into the solution to verify the pH. Then distilled water was added to the solution until the volume reached 2L.

The solution was poured into four 1L Erlenmeyer flasks so that each contained 500 ml of the solution. (It is important that the depth of the liquid does not exceed roughly 1.5 inches to ensure that the autoclaving conditions reach the center). The openings of the flasks were sealed with aluminum foil and pieces of autoclave tape were placed on top of the foil.

The flasks were put into an autoclave bin, the bin was placed inside an autoclave, and the door was closed tightly. The cycle time was set at 15 Sterilize minutes (Dry time = 0). The orange Fluid Cycle button and then the red "On" button were pressed. The autoclave pressurized to 15 psi and the cycle began when the green and red arrows align at 121°C.

Once the 15 minute cycle was completed, there was an additional wait of 10-15 minutes to allow the pressure and heat to come down from the cycle. Then the door was slowly opened (to allow for careful steam release). Bins were removed from the autoclave using thermally insulated gloves.

The flasks were set aside at room temperature for inoculation the following day.

3.2.8 Dilution Series

A dilution series was performed to determine and verify the viability of the *E*. *coli*. Three dilution bottles were each filled with 99 ml of Butterfield's Solution. The lids were put on the bottles but left loose so that pressure could release during autoclaving. Autoclave tape was adhered to the tops of the lids. The bottles were placed in a wire basket and the basket was placed in an autoclave bin. The autoclaving procedure for Butterfield's Solution was followed. The bottles were labeled 1, 2, and 3, respectively.

To perform the dilution series, 1 ml of *Escherichia coli* K12 ATCC Number 29181 stock was needed. The *E. coli* was stored in vials in a -80°C freezer (Figure 19). Each vial contained approximately 1 ml of *E. coli*. One vial was removed from the freezer and thawed by rolling it in between hands; this also ensured the *E. coli* was well mixed.



Figure 19. Vials of *E. coli* stock.

A 1 ml pipette with disposable pipette tips was used to transfer the *E. coli* into bottle 1. A sterile tip was placed on the pipette and then 1 ml of the *E. coli* was drawn up into the pipette from the vial. The lid on the bottle was carefully removed and held in one hand while maintaining its orientation. With the other hand, the pipette was lowered into the bottle until its tip was in the solution. Then the *E. coli* was pushed from the pipette into the solution. (During this procedure, it is important the sides of the pipette do not come in contact with the sides of the bottle so that contamination does not occur). The pipette was lifted from the bottle and the tip disposed of. Then the cap was placed tightly back on top of the bottle and the bottle was shaken for 30 seconds at a 1 ft length to mix the *E. coli* in the solution. Bottle 1 had dilution factor of 10^{-2} .

Another sterile tip was placed on the 1 ml pipette, and following the same methodology, 1 ml of solution was pulled into the pipette from bottle 1 and transferred to bottle 2. Bottle 2 was also shaken for 30 seconds at a 1 ft length to ensure mixing. Bottle 2 had a dilution factor 10^{-4} . This procedure was repeated a third time to transfer 1 ml of solution from bottle 2 to bottle 3 so that bottle 3 had a dilution factor of 10^{-6} .

To continue with the dilution series, aliquots of solution from the bottles had to be placed on agar plates. The six agar plates were arranged in a row and labeled from left to right so that each one was labeled with one of the following dilution ratios: 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} . It is more efficient to perform the series from right to left (10^{-7} to 10^{-2}) because the same pipette can be used for the entire process. Using a sterile

pipette, 0.1 ml of solution was drawn up from bottle 3 and added to plate 10⁻⁷. Then 1 ml of solution from bottle 3 was drawn up and added to plate 10⁻⁶. From bottle 2, 0.1 ml was drawn up and added to plate 10⁻⁵, and 1 ml was drawn up and added to plate 10⁻⁴. From bottle 1, 0.1 ml was drawn up and added to plate 10⁻³, and 1 ml was drawn up and added to plate 10⁻² (Figure 20).



Figure 20. Dilution series.

Lastly, the solution was spread on the plates. Setup for this included a boat containing 70% isopropyl alcohol and a lit Bunsen burner. A glass spreader ("hockey stick") was dipped into the alcohol and flamed with the burner. (It is important to keep the end of the stick that was dipped in alcohol tipped downward so that alcohol cannot drip toward the hand). Working again from 10⁻⁷ to 10⁻², the stick was used to spread the solution over each agar plate in up-and-down, side-to-side, and around-the-edge motions to ensure even coating. The plates were incubated at 35°C, 50% RH for 24 hours.

After the incubation period was complete, the colony forming units (CFU) on each plate were counted to determine the viable concentration of the stock. A Quebec Colony Counter (Figure 21) was used to light and magnify, and a Sharpie was used to mark the plate as each colony was counted.



Figure 21. Quebec Colony Counter illuminating a dilution series agar plate.

3.2.9 Inoculation

Prior to working in the lab, all table tops were disinfected using Multiterge (5 ml in 100 ml of water). To inoculate the Butterfield's Solution, and as determined by the dilution series, 1 ml of *Escherichia coli* K12 ATCC Number 29181 was required for each 100 ml volume of solution; therefore, 5 ml of *E. coli* were added to each flask containing 500 ml of solution. This produced a 1×10^6 cells/ml microbial solution concentration.

The vials of *E. coli* were removed from the freezer and thawed by rolling them in between hands. Each vial contained approximately 1 ml of *E. coli*. A 1 ml pipette with disposable pipette tips was used to transfer the *E. coli* into the flasks containing sterile Butterfield's Solution. A sterile tip was placed on the pipette and then 1 ml of the *E. coli* was drawn up into the pipette from a vial. The foil on the flask was carefully removed and set aside so that its orientation remained the same; this helps reduce the risk of contaminating the foil. The pipette was lowered into the flask until its tip was in the solution. Then the *E. coli* was pushed from the pipette do not come in contact with the sides of the flask so that contamination does not occur). The pipette was lifted from the flask and the tip disposed of. This procedure was repeated until the flask had 5 ml of *E. coli* added to it. Then the foil was placed back on top of the opening and the flask was gently agitated to mix the *E. coli* in the solution. This procedure was repeated for all flasks.

3.2.10 Microbial Challenge and Induction of Pressure Differential

A microbial challenge chamber consisting of 0.25 in thick Plexiglas was used in this study (Figures 22 and 23). The internal dimensions of the chamber are 28.5 in x 20 in x 36 in (L x W x H). The chamber was designed with one removable face attached with wing nuts; this allows entry into the cabinet for sample loading and chamber cleaning. This face also contains a pair of gloves used for manipulation of objects inside the chamber while maintaining the seal of the chamber (Figure 23). The interior of the chamber contains a racking system that holds four samples. This racking system also holds four syringes (for induction of pressure differential) and includes a retraction bar that can be used to simultaneously draw back the syringes. The racking system is approximately 12 inches from the floor of the chamber. Two paint sprayers are affixed to the top of the chamber to aerosolize the microbial solution (Figure 22). The sprayers are Central Pneumatic Air Spray Guns Model #43760 with internal mix nozzles measuring 0.082 inches in diameter. For this study, they were pressurized at 40 psi (Severin 2006).



Figure 22. Microbial challenge chamber.



Figure 23. Interior view of microbial challenge chamber.

Prior to microbial challenge testing, the chamber was cleaned using a bleach solution. Additionally, the two spray canisters were thoroughly rinsed with water, including spraying water from them to rinse the spray nozzles. Each canister was then filled with 1L of the microbial solution and affixed to the top of the chamber. The canisters were attached to air hoses with pressure set at 40 psi.

Next, the 60 ml syringes (maximum of four) used for induction of pressure differential (Becton, Dickinson and Company, Franklin Lakes, NJ) were loaded into the chamber. Each was outfitted with an 18G1½ needle (Becton, Dickinson and Company, Franklin Lakes, NJ) with the cap still intact to maintain sterility. Test samples were then individually loaded into the chamber in batches of four (Figure 23). First, the pressure differential septum on the exterior of the test sample was sterilized with an alcohol swab. Then the cap on the needle was removed from a syringe secured in the chamber so that it could pierce through the septum on the sample as the sample was placed in its position in the chamber (Figure 24). It was important to ensure accurate alignment of the needle through the septum(s) into the tray so that it remained above the agar in the headspace. It was also important to ensure that the needle did not accidentally puncture another surface of the package during insertion.



Figure 24. Pressure differential syringe placement.

Four samples can fit into the chamber for each test run. Once all samples were loaded, the retraction bar used to simultaneously and consistently pull the syringe plungers back to induce pressure differential was placed on the plunger handles. (Figure 25). Lastly, an open agar plate was placed on the bottom of the chamber (Figure 25) to verify the viability of the microbial solution and spray consistency across the chamber. Sample position and run were recorded.



Figure 25. Retraction bar and agar plate placement.

Once the samples and agar plate were in place, the chamber was closed and ready for microbial challenge. The canisters were sprayed simultaneously for 15 seconds. Then the retraction bar on the syringes was pulled to induce pressure differential. This was done by placing hands in the gloves affixed to the chamber, and consistently pulling the bar back for approximately one minute until the syringes had pulled a volume of 62 ml of air from the test samples. This induced a pressure differential of -3.78 psi, which is equivalent to an aircraft or ground shipment descent of 8,000 ft (Severin 2006).

Thirty minutes from the time the microbial solution was aerosolized into the chamber, the syringes were removed from the samples and the samples were transferred to an environmental chamber for incubation. Additionally, the agar plate for each run was incubated.

This entire microbial challenge process was repeated until all samples were tested. Upon completion, the chamber was cleaned with a bleach solution and the canisters were thoroughly rinsed with water.

3.2.11 Incubation

After the microbial challenge testing, the samples and agar plates were incubated in an environmental chamber at 37°C and 50% RH for approximately 48 hours.

3.2.12 Colony Counting of Test Samples

Test samples were inspected for colony growth after the incubation period was complete. The bases of the inverted trays were cut away using a razor blade to create a viewing window (Figure 26). If colonies were present, they were counted and recorded. (See Appendix C for results table.)



Figure 26. Colony viewing window cut from tray.

3.2.13 Colony Counting of Agar Plates

After the incubation period, colonies were also counted on the agar plates to verify the viability of the microbial solution. A Quebec Colony Counter (Figure 27) was used to light and magnify, and a Sharpie was used to mark the plate as each colony was counted. The number of CFU was recorded. (See Appendix D for results table.)



Figure 27. Quebec Colony Counter illuminating a microbial challenge agar plate.

3.2.14 Enterotubes

Enterotubes (BD BBL[™] Enterotube[™] II REF 211832) were used to verify that the observed CFU were *Escherichia coli* K12 ATCC Number 29181. The white cap of the tube was removed to expose a wire that was dipped into the colony being tested and the cap was replaced. Following this, the blue cap was removed from the opposite end of the tube to expose a wire handle. The handle was used to pull the wire (while gently twisting it back and forth) so that the end that touched the colony traveled through each compartment of the tube. Then the wire was pushed back through the tube

towards its starting position until the notch on the wire aligned with the edge of the tube. The wire was bent back and forth to break it off at the notch and the blue cap was replaced. The piece of wire that was broken off was used to pierce holes through the air inlets of the compartments that had them. The tubes were incubated at 35°C, 50% RH for 18 hours.

After the incubation period was complete, the enterotubes were observed. Wearing latex gloves, Kovac's reagent was drawn up in a 1 ml syringe and five drops were injected into each Indole compartment. Then the tubes were color matched to a key to verify that the colony was the test organism (Figure 28).



Figure 28. Example of enterotube and color key.

3.3 Results and Discussion

Two dependent variables were considered during analysis: the number of trays that exhibited growth (binary data: growth or no growth), and the number of CFU observed per tray (variable data).

A total of 30 usable trays were tested for the effect of pressure differential on microbial ingress through a 100 μ m pinhole in sterile trays. Of the fifteen trays that were exposed to a pressure differential, all of them (100%) exhibited growth (Figure 29). Of the fifteen trays that were not exposed to a pressure differential, nine (60%) exhibited growth (Table 5). (See Appendix C for results table).



Figure 29. Example of tray with colony growth.

Pressure Differential (psi)	Sample Size	Samples with Growth	% Growth	CFU Mean	CFU Standard Error Mean
-3.78	15	15	100%	21.0667	4.8745
0	15	9	60%	1.0667	0.3584

Table 5. Percent of total trays with CFU.

Question 1: What is the effect of pressure differential on the probability of microbial penetration of medical device trays? (Binary data: growth or no growth)

The response variable, trays with microbial ingress, was modeled as binary

distribution using a generalized linear mixed model fitted with the GLIMMIX procedure

of SAS (SAS version 9.1, SAS Institute Inc., Cary, NC). Due to extreme category problems (all trays assigned to pressure differential had growth), the effect of pressure was modeled as random in a Bayesian-type approach. Sensitivity analyses were performed using starting values for the random variance equal to 1, 10, 100, 1000, and 3000. Variance estimates and pressure differences did not change for the starting values considered. Contrasts were used to compare levels of pressure. Other random factors such as sealing run, sealing position, injection run, injection frame position, chamber run, and chamber position were evaluated for inclusion in the model. Due to variance components estimates equal to 0, these random blocks were not included in the final model. The null and research hypotheses for this analysis are:

- H₀: Pressure differential will not increase the number of trays with microbial ingress.
- H_{a:} Pressure differential will increase the number of trays with microbial ingress.

Here, the null hypothesis was not rejected; therefore, conclusions cannot be made on the research hypothesis. A marginal effect of pressure differential (p=.0694) on the probability of microbial ingress in trays was identified (α =.05). The predicted probabilities of microbial penetration were 87.1 ± 26.8% for pressure differential and 12.9 ± 26.8% for no pressure differential (Figure 30).



Figure 30. Comparison of predicted probabilities of microbial penetration when pressure differential is the independent variable.

Question 2: What is the effect of pressure differential on the number of CFU in trays? (Variable data: number of CFU)

A generalized linear mixed model was fitted using a negative binomial distribution of the response variable, number of CFU, using the GLIMMIX procedure of SAS (SAS version 9.1, SAS Institute Inc., Cary, NC). The model included the fixed effect of pressure. Due to convergence problems, it was not possible to include other random blocking factors such as sealing run, sealing position, injection run, injection frame position, chamber run, and chamber position in the final model. The null and research hypotheses for this analysis are:

- H₀: Pressure differential will not increase the number of CFU in trays.
- H_a: Pressure differential will increase the number of CFU in trays.
Here, the null hypothesis was rejected and the research hypothesis was accepted. Data provided evidence for a significant effect of pressure differential (p <.0001) on the number of CFU in trays (α =.05); there was a significantly greater number of CFU in trays exposed to a pressure differential than without. The estimated number of CFU was 21.1 ± 4.9 for trays exposed to a pressure differential, and 1.1 ± 0.3 for no exposure (Figure 31).



Figure 31. Comparison of estimated number of CFU per tray when pressure differential is the independent variable.

PART 2

EFFECTS OF PRESSURE DIFFERENTIAL AND SECONDARY PACKAGING ON MICROBIAL INGRESS OF STERILE MEDICAL DEVICE TRAYS

4.1 Objective

The objective for Part 2 was:

• to examine the effects of secondary packaging (pouches and cartons) and

pressure differential on the microbial ingress of sterile medical device trays.

4.2 Materials and Methods

The methods employed to investigate all objectives were the same as in Part 1

(except where indicated) and included the following steps:

1. aseptically filling sealed, sterile device packages with a known volume of

appropriate sterile growth medium,

- 2. exposing the packages to an aerosolized microbial challenge,
- inducing pressure differential on the packages inside the microbial challenge chamber,
- 4. incubating the packages,
- 5. inspecting for growth,
- and recording binary (growth or no growth) and variable (number of colony forming units) data.

A total of one-hundred and twenty samples were tested to investigate the study objective. Forty of the samples were unlidded trays sealed in Nylon/LDPE/HDPE

pouches, forty were unlidded trays packaged in paperboard cartons, and the remaining forty were lidded trays, each containing a 100 µm pinhole and sealed with a nonporous lid, packaged in paperboard cartons. All samples were uniquely identified and aseptically injected with sterile growth medium. Of these, usable data was obtained from 118: 39 unlidded trays in pouches, 39 unlidded trays in cartons, and 40 lidded trays, each containing a 100 µm pinhole, in cartons. The "unlidded tray in pouch" sample that was removed from the study had the pouch punctured by the syringe used to induce pressure differential. The "unlidded tray in carton" sample that was removed from the study had the syringe used to induce pressure differential pull out of the sample during that procedure.

The samples were subjected to an aerosolized microbial challenge with a solution of *Escherichia coli* K12 ATCC Number 29181 that had a starting concentration of 1×10^{6} cells/ml. The aerosol was sprayed for 15 seconds and allowed to settle for 30 minutes before the samples were removed from the chamber. Immediately following aersolization, a pressure differential was induced on all of the test samples by withdrawing a known volume of air from the headspace of all of the packages with a needle and syringe. Run number and sample position within the chamber were recorded.

Independent variables for model consideration included: injection number, chamber run, chamber position, lidding condition (tray lidded or unlidded), secondary package type (pouch or carton), and the presence or absence of a 100 µm laser-drilled hole. The design approach was tiered, first investigating the most "open" designs

(unlidded) to determine if penetration could occur with the secondary packages and then adding lids from there to determine if penetration could occur through both the secondary and primary packages in conjunction.

The 40 unlidded trays in pouches were exposed to the aerosolized microbial solution and pressure differential in ten runs of four trays each. No growth was observed in any of the 39 usable samples; therefore, there was no additional investigation of lidded trays in pouches.

The 40 unlidded trays in cartons were also exposed to the aerosolized microbial solution and pressure differential in ten runs of four trays each. Because 37 out of 39 usable samples exhibited growth, an additional tier of testing was added (lidded trays with 100 μm pinholes in cartons).

The 40 trays sealed with nonporous lids, each containing a 100 μ m pinhole and packaged in paperboard cartons, were also exposed to the aerosolized microbial solution and pressure differential in ten runs of four trays each.

4.2.1 Sample Preparation for Unlidded Trays in Pouches

The trays each had a self-sealing septum (Mocon, Minneapolis, MN) adhered to them on the front corner; these septums later served as the sites used for inducing pressure differential (Figure 32).



Figure 32. Unlidded tray with pressure differential septum.

Test pouches were 100GA Biax Nylon (0.001), 0.0007 LDPE, 0.002 HDPE Coex pouches (Lot #: H150978/1/A) with dimensions of 7.25 in x 9.50 in (Mangar Industries, Inc., New Britain, PA) (Figure 33). One unlidded tray was placed inside each pouch.



Figure 33. Example of test pouch.

Pouches were sealed using a CeraTek model 24-AS/1 (Serial No. 06-04236) heat bar sealer (SenCorp, Hyannis, MA). Sealing parameters were: 275°F, 60 psi, and 1 second of dwell time. After sealing, pouches were visually inspected for defects according to ASTM F 1886 Standard Test Method for Determining Integrity of Seals for Medical Packaging by Visual Inspection (ASTM 2004); those with identified seal defects were removed from the study. The remaining samples each had a self-sealing septum (Mocon, Minneapolis, MN) adhered to the top exterior of the pouch over the open top of the tray; these septums later served as the agar injection sites (Figure 34). Additionally, each pouch had a self-sealing septum adhered to the bottom exterior so that it aligned with the pressure differential septum on the tray (Figure 34). Each pouch was placed in a paperboard carton (to protect from distribution hazards) and the cartons were packed into corrugated shippers for sterilization. These cartons were for transportation only and were removed prior to agar injection and microbial challenge testing.



Figure 34. Unlidded tray in pouch test configuration with septum placement.

4.2.2 Sample Preparation for Unlidded Trays in Cartons

Reverse tuck folding cartons were constructed from 15.5 pt solid bleached sulfate (SBS) on an Artios Kongsberg Premium Line 1930 cutting table. Carton dimensions were 5 3/16 in x 15/16 in x 6 3/4 in.

The trays each had a self-sealing septum (Mocon, Minneapolis, MN) adhered to the front corner; these septums later served as the sites for induction of pressure differential (Figure 35). A single tray was placed inside each carton so that all samples were in the same orientation. The cartons were labeled with the tray identification numbers.

It was imperative that all septums placed on the cartons aligned with the septums on the trays inside. To ensure this, a template was used to identify placement points for the septums and injection points on the septums. The template, constructed from one of the cartons, was used to identify the center points on the tops of the test cartons. At each center point, a self-sealing septum was adhered to the top exterior of the carton over the open top of the tray; these septums later served as the agar injection sites (Figures 35 and 36). Additionally, each carton had a self-sealing septum on the tray (Figure 36).



Figure 35. Figure 36. Unlidded tray in carton test configuration with septum placement: open view (Figure 35), closed view (Figure 36).

Lastly, the template was used to mark the injection points on the pressure differential septums with ink (Figure 37). Since it was not possible to see the trays inside, this was done to ensure that the needles would align and pierce through both septums. The cartons were packed into corrugated shippers for sterilization.



Figure 37. Injection points on pressure differential septums.

4.2.3 Sample Preparation for Lidded Trays, with 100 μm Pinholes, in Cartons

Lidded trays, each containing one 100 µm pinhole, were visually inspected for damage to the trays, lids, and seals. Each tray had a self-sealing septum (Mocon, Minneapolis, MN or Illinois Instruments, Johnsburg, IL) adhered to the center of the lid that later served as the agar injection site. The trays also had a self-sealing septum adhered to them at the front corner to later serve as the site used for inducing pressure differential (Figure 38). Here, the opposite corner is used because the trays are inverted (with their lid sides down) inside the microbial challenge chamber; this ensured that the fixtures for inducing pressure differential inside the chamber would still align with the septums on the samples.



Figure 38. Septum placement for lidded tray in carton test configuration.

One tray was placed inside each carton so that all samples were in the same orientation (lid side down) and the cartons were labeled with the tray identification numbers (Figure 39).



Figure 39. Lidded tray in carton test configuration.

The template was again used to identify the locations for adhering septums to the cartons and marking the septums with the injection points. Here, the agar injection septums were placed on the bottom exterior of the cartons because the trays were lid side down inside the cartons (Figure 40). Lastly, the cartons were packed into corrugated shippers for sterilization.



Figure 40. Figure 41. Lidded tray in carton septum placement: bottom view (Figure 40), top view (Figure 41).

4.2.4 Agar Injection for Unlidded Trays in Pouches and Unlidded Trays in Cartons

The agar injection process for unlidded trays in pouches and unlidded trays in cartons, including syringe and needle setup and drawing up of the agar into the syringe, were performed as stated previously with the lidded tray injection. The samples were visually inspected for any defects; those with defects were removed from the study. Each sample was placed and oriented on the lab bench so that the opening of the tray faced up. Injection number was recorded. The septum on the top of the pouch or carton was disinfected using a 70% isopropyl alcohol swab. The vented needle was pushed through the septum at an angle so that it did not pierce through the bottom of the tray inside. The agar was slowly pushed out of the syringe into the tray or pouch (Figure 42) or carton. Once the entire volume of agar was injected, the syringe was removed from the sample. The sample was gently agitated so that the agar covered the bottom surface of the tray evenly.



Figure 42. Example of agar injection into unlidded tray in pouch configuration.

If any errors occurred during this procedure (inadvertent puncture, etc.), those samples were removed from the study. All samples were stacked in corrugated boxes and held at room temperature until the following day for the microbial challenge procedure.

4.2.5 Agar Injection for Lidded Trays, with 100 μm Pinholes, in Cartons

Cartons containing lidded trays with 100 µm pinholes that were injected with sterile agar each went through the following process: The sample was visually inspected for any defects; those with defects were removed from the study. The sample was placed and oriented on top of the injection frame with the lid-side down so that trays containing holes had the hole-side at the top and the holes could not become blocked with agar (Figure 43). Injection number was recorded. The septum on the bottom of the carton was disinfected using a 70% isopropyl alcohol swab.



Figure 43. Test sample placement on agar injection frame (bottom view).

The agar injection process, including syringe and needle setup and drawing up of the agar into the syringe, were performed as stated above with the lidded tray injection. The vented needle was pushed through the septum on the carton, that aligned with the septum on the tray lid inside, at an angle so that it did not pierce through the other side of the tray and carton. The agar was slowly pushed out of the syringe into the tray so that it filled the bottom surface and did not touch the other surfaces inside the tray. Once the entire volume of agar was injected, the syringe was removed from the sample. The sample was gently agitated so that the agar covered the bottom surface of the tray evenly.

If any errors occurred during this procedure (inadvertent punctures, etc.), those samples were removed from the study. All samples were stacked in corrugated boxes and held at room temperature until the following day for the microbial challenge procedure.

4.2.6 Microbial Challenge and Induction of Pressure Differential

Test samples were subjected to microbial challenge, induction of pressure differential, and incubation using the methods described in Part 1. The same methods in Part 1 were also employed for colony counting of agar plates (see Appendix E for results tables) and use of enterotubes.

4.2.7 Colony Counting of Test Samples

Test samples were inspected for colony growth after the incubation period was complete. For test sample configurations with open trays in pouches or cartons, the trays were removed from the secondary package and visually inspected for colony

growth on the agar. If colonies were present, they were counted and recorded. (See Appendix F for results tables).

For test sample configurations that had lidded trays inside cartons, the trays were removed from the cartons and the bases of the inverted trays were cut away using a razor blade to create a viewing window. If colonies were present, they were counted and recorded. (See Appendix F for results table.)

4.3 Results and Discussion

Two dependent variables were considered during analysis: the number of trays that exhibited growth (binary data: growth or no growth), and the number of CFU observed per tray (variable data).

A total of 118 usable trays were tested for the effect of secondary packaging (pouches and cartons) on microbial ingress of sterile trays (lidded and unlidded). Zero (0%) of the 39 "unlidded tray in pouch" samples exhibited growth. Of the 39 "unlidded tray in carton" samples, 37 (95%) exhibited growth. Of the 40 "lidded tray in carton" samples, 6 (15%) exhibited growth (Table 6). (See Appendix F for results tables.)

Sample Type	Sample Size	Samples with Growth	% Growth
Unlidded tray in pouch	39	0	0%
Unlidded tray in carton	39	37	95%
Lidded tray, with 100 μ m pinhole, in carton	40	6	15%

Table 6.	Percent of to	tal trays	s with	CFU.
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Question 1: What is the effect of secondary package type on the probability of microbial penetration of trays? (Binary data: growth or no growth)

The response variable, microbial penetration, was modeled as binary distribution using a generalized mixed model fitted with the GLIMMIX procedure of SAS (SAS version 9.2, SAS Institute Inc., Cary, NC). The model included the fixed effect of treatment (secondary package type) and the random effect of run nested within treatment, in order to account for technical replication present in the design. The marginal log likelihood was approximated using an adaptive Gauss-Hermite quadriture method in order to expedite model convergence. Due to an extreme category problem (quasicomplete separation of data points given by no penetration in any of the trays assigned to pouches), the effect of treatment was modeled as a random effect in a Bayesian-type approach. Sensitivity analyses were performed using starting values for the random variance equal to 1, 10, 100, 1000, and 3000. Variance estimates and treatment differences were not affected by the starting values considered; therefore, the Bayesian analysis was considered robust and valid. Contrasts were used to compare treatments. The null and research hypotheses for this analysis are:

- H₀: There will be no difference in the probability of microbial penetration between trays packaged in pouches and trays packaged in cartons.
- H_a: Trays packed in pouches will have a lower probability of microbial penetration than trays packaged in cartons.

Here, the null hypothesis was rejected and the research hypothesis was accepted. Data provided evidence of a significant effect of secondary package type (p=.0102) on microbial penetration of trays (α =.05). The type of secondary package (pouch or carton) affected the probability of microbial penetration of trays, such that penetration was more likely to occur in trays packed inside cartons (99.96% predicted probability) compared to trays packaged inside pouches (0%) when the trays were unlidded (Figure 44).



Figure 44. Comparison of predicted probability of microbial penetration of pouches and cartons.

Question 2: What is the effect of secondary package type on the number of CFU in

trays? (Variable data: number of CFU)

The response variable, number of CFU, was modeled as Poisson distributed with

overdispersion using a generalized linear mixed model fitted with the GLIMMIX

procedure of SAS (SAS version 9.2, SAS Institute Inc., Cary, NC). The model included the

fixed effect of treatment (secondary package type) and the random effect of run nested

within treatment in order to account for technical replication present in the design. Due to all zero-counts in one of the treatments, convergence was impaired and the effect of treatment was modeled as a random effect in a Bayesian-type approach as with Question 1. Contrasts were used to compare treatments. The null and research hypotheses for this analysis are:

- H₀: There will be no difference in the number of CFU between trays packaged in pouches and trays packaged in cartons.
- H_a: Trays packed in pouches will have less CFU than trays packaged in cartons.

Here, the null hypothesis was rejected and the research hypothesis was accepted. Data provided evidence of a significant effect of secondary package type (p=.0279) on the number of CFU in trays (α =.05). Type of secondary package affected the number of CFU in trays such that the number of CFU was greater in trays packed inside cartons (18.7) compared to trays packaged inside pouches (0.1) (Figure 45).



Figure 45. Comparison of estimated number of CFU per tray in pouches and cartons.

Question 3: Given a secondary package of a carton, does the presence of a lid affect the probability of microbial penetration of trays? (Binary data: growth or no growth)

The response variable, microbial penetration, was modeled as binary distribution using a generalized mixed model fitted with the GLIMMIX procedure of SAS (SAS version 9.2, SAS Institute Inc., Cary, NC). The model included the fixed effect of treatment (presence or absence of a lid) and the random effect of run nested within treatment, in order to account for technical replication present in the design. The marginal log likelihood was approximated using an adaptive Gauss-Hermite quadrature method in order to expedite model convergence. The null and research hypotheses for this analysis are:

- H_0 : When packaged inside cartons, there will be no difference in the probability of microbial penetration between trays with lids and a 100 μ m hole and trays without lids.
- H_a: When packaged inside cartons, trays with lids and a 100 μm hole will have a lower probability of microbial penetration than trays without lids.

Here, the null hypothesis was rejected and the research hypothesis was accepted. Data provided evidence of a significant effect of lidding condition (p<.0001) on the likelihood of microbial penetration in trays packaged inside cartons (α =.05). Microbial penetration was more likely to occur in trays without a lid (94.9 ± 3%) compared to those with a lid and a 100 µm hole (15 ± 5%) (Figure 46).



Figure 46. Comparison of predicted probability of microbial penetration of unlidded and lidded trays in cartons.

Question 4: Given a secondary package of a carton, does the presence of a lid affect the number of CFU in trays? (Variable data: number of CFU)

The response variable, number of CFU, was modeled as Poisson distributed with overdispersion using a generalized linear mixed model fitted with the GLIMMIX procedure of SAS (SAS version 9.2, SAS Institute Inc., Cary, NC). The model included the fixed effect of treatment (presence or absence of a lid) and the random effect of run nested within treatment in order to account for technical replication present in the design. The null and research hypotheses for this analysis are:

• H_0 : When packaged inside cartons, there will be no difference in the number of CFU between trays with lids and a 100 μ m hole than trays without lids.

 H_a: When packaged inside cartons, trays with lids and a 100 μm hole will have less CFU than trays without lids.

Here, the null hypothesis was rejected and the research hypothesis was accepted. Data provided evidence of a significant effect of lidding condition (p=.0048) on the number of CFU present in trays packaged inside cartons (α =.05). The number of CFU was greater in trays without a lid (14.6 ± 4.4) compared to those with a lid (0.1 ± 0.2) (Figure 47).



Figure 47. Comparison of estimated number of CFU per tray of unlidded and lidded trays in cartons.

PART 3

EFFECTS OF HOLE SIZE, PRESSURE DIFFERENTIAL, AND SECONDARY PACKAGING ON MICROBIAL INGRESS OF STERILE MEDICAL DEVICE TRAYS

5.1 Objective

The objective for Part 3 was:

 to examine and compare the combined effects of hole size, pressure differential, and secondary packaging on the microbial ingress of lidded sterile medical device trays.

5.2 Materials and Methods

The methods employed to investigate the objective were the same as in Parts 1

and 2 (except where indicated) and included the following steps:

- aseptically filling sealed, sterile device packages with a known volume of appropriate sterile growth medium,
- 2. exposing the packages to an aerosolized microbial challenge,
- inducing pressure differential on the packages inside the microbial challenge chamber,
- 4. incubating the packages,
- 5. inspecting for growth,
- and recording binary (growth or no growth) and variable (number of colony forming units) data for use as the dependent response variables.

The experimental design is a split plot with three factors containing two levels each:

- Factor A: pressure differential (0 and -3.78 psi)
- Factor B: hole size (10 and 100 μm)
- Factor C: secondary packaging (presence or absence of a paperboard carton).

Each test sample contained one level of each of the three factors so that there were eight configurations total (Figure 48). Trays contained either a 10 or 100 μ m pinhole, were packaged inside a carton or without a carton, and were subjected to 0 or -3.78 psi of pressure differential. The eight configurations were:

- 1. 10 µm hole, carton, 0 psi
- 2. 10 µm hole, no carton, 0 psi
- 3. 10 µm hole, carton, -3.78 psi
- 4. 10 μm hole, no carton, -3.78 psi
- 5. 100 µm hole, carton, 0 psi
- 6. 100 μm hole, no carton, 0 psi
- 7. 100 μm hole, carton, -3.78 psi
- 8. 100 µm hole, no carton, -3.78 psi

0 psi		-3.78 psi		
Carton	No Carton	Carton	No Carton	
1	2	3	4	10 µm
5	6	7	8	100 µm

Figure 48. Eight test sample configurations combining levels of hole size, presence or absence of a carton, and presence or absence of a pressure differential.

The microbial challenge chamber was limited to four samples per run; therefore, the whole plot factor was pressure differential (Factor A) with the experimental unit being chamber run, or block. The split-plot factors were hole size and secondary packaging (Factors B and C, respectively) with the experimental units being each treatment combination, or observation.

Each run of the microbial challenge chamber was assigned one of the two levels of pressure differential (Factor A). Within the chamber, test samples contained one level each of Factors B and C (hole size and presence or absence of a carton). The four chamber positions were identified as A, B, C, or D (Figures 49 and 50). The position of the test samples within the chamber was randomized, but it is notable that previous studies (Severin 2006) and the preliminary research presented herein did not suggest an effect of chamber position on microbial ingress of the trays.

А	В	С	D
10 μm	10 μm	100 μm	100 μm
Carton	No Carton	Carton	No Carton

Figure 49. Microbial challenge chamber (assigned to either 0 or -3.78 psi) containing four test samples (assigned to combinations of hole size and presence or absence of a carton).



Figure 50. Microbial challenge chamber (assigned to -3.78 psi) containing four test samples (assigned to combinations of hole size and presence or absence of a carton).

Power analyses were conservatively performed to identify the necessary sample sizes for both the predicted probability of microbial ingress and the number of CFU as response variables when α =.05. Main effects (pressure differential, hole size, and secondary package), their 3-way interaction, and the contrast of the research hypotheses extremes (100 µm hole, -3.78 psi pressure differential, and no carton vs. 10 µm hole, 0 psi pressure differential, and carton) were analyzed.

To have a power of 80% for both response variables, approximately 80 chamber runs of four trays each were required for all effects except the 3-way interaction when predicted probability of microbial ingress is the response variable (Figures 51 and 52). Here, the analysis indicates that 200 runs of four trays each results in a power of 65% (Figure 63). As a result, 320 samples (80 runs of four) were required to reach 80% power for all effects excluding the 3-way interaction.



Figure 51. Power analysis for predicted probability of microbial ingress.



Figure 52. Power analysis for number of CFU.

All samples were uniquely identified. Of the 320 samples, 304 were usable, resulting in 76 runs of the chamber (see Appendix G for agar plate colony count table). There were 38 samples for each of the 8 test configurations. Samples that were removed from the study were damaged during the agar injection process.

5.3 Results and Discussion

Two dependent variables were considered during analysis: the number of trays that exhibited growth (binary data: growth or no growth), and the number of CFU observed per tray (variable data).

A total of 304 usable trays were tested for the effects of hole size, pressure differential, and presence or absence of a carton, with 38 samples for each test configuration. The number of samples that exhibited growth and the percent growth for each sample type are presented in Table 7 and Figure 53. (See Appendix H for

results table.)

Sample Type	Sample Size	Samples with Growth	% Growth
10 μm, carton, 0 psi	38	0	0%
10 μm, no carton, 0 psi	38	0	0%
10 μm, carton, -3.78 psi	38	1	3%
10 μm, no carton, -3.78 psi	38	3	8%
100 μm, carton, 0 psi	38	2	5%
100 μm, no carton, 0 psi	38	14	37%
100 μm, carton, -3.78 psi	38	29	76%
100 μm, no carton, -3.78 psi	38	35	92%

Table 7. Percent of total samples with CFU.

0 psi		-3.7		
Carton	No Carton	Carton	No Carton	
0%	0%	3%	8%	10 µm
5%	37%	76%	92%	100 µm



Question 1: What are the combined effects of hole size, pressure differential, and cartons on the probability of microbial penetration of trays? (Binary data: growth or no growth)

A generalized linear mixed model was fitted to the binary response variable indicating whether growth was observed in the test samples $(0 = n_0, 1 = y_0)$. The statistical model used for analysis included the fixed effects of pressure differential (0 and -3.78 psi), hole size (10 and 100 μ m), presence of a carton (1 = yes, 2 = no), and the 2-way interactions between pressure differential and carton, and between hole size and carton. The remaining interaction terms, the 2-way interaction between pressure differential and hole size and the 3-way interaction between all three effects, were considered for inclusion in the model but were dropped due to model overfit and pvalues >.90. The model also included the random effect of chamber nested within pressure differential in order to recognize the experimental unit for this factor and the split plot arrangement of the experimental design. The fixed effect of chamber position (A, B, C, D) was also considered in the model to check the randomization process. Chamber position was found to be not significant (p = .15) and was dropped from the model. Degrees of freedom were estimated using Kenward Roger's procedure. The model was fitted using the GLIMMIX procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC). Estimated probabilities of contamination for combinations of the factors of interest, as well as differences between factor levels, were reported. Pairwise comparisons were conducted using Tukey-Kramer adjustment to avoid inflation of Type I error rate due multiple comparisons.

The null and research hypotheses for this analysis are:

- H₀: There will be no difference in the probability of microbial penetration when comparing any of the test configurations.
- H_a: Trays containing a 10 μm hole, in the presence of a carton, and in the absence of a pressure differential will have the lowest probability of microbial penetration while trays containing a 100 μm hole, in the absence of a carton, and in the presence of a pressure differential will have the highest probability of microbial penetration.

The data did not support any 3-way or any 2-way interaction between the 3 factors of interest. A main effect of pressure differential was identified (p<.0001, α =.05). The probability of contamination was significantly increased under conditions of a negative pressure differential (0.53 ± 0.08) compared to no pressure differential (0.01 ± 0.01) (Figure 54).



Figure 54. Comparison of probabilities of microbial penetration when pressure differential is the main effect.

A main effect of hole size was identified (p<.0001, α =.05). The probability of contamination was significantly increased with 100 µm holes (0.51 ± 0.07) compared to 10 µm holes (0.007 ± 0.005) (Figure 55).





A main effect of secondary packaging was identified (p=.0146, α =.05). The

probability of contamination was significantly increased in the absence of a carton (0.18

 \pm 0.05) compared to the presence of a carton (0.04 \pm 0.02) (Figure 56).



Figure 56. Comparison of probabilities of microbial penetration when secondary packaging is the main effect.

Question 2: What are the combined effects of hole size, pressure differential, and cartons on the number of CFU in trays? (Variable data: number of CFU)

A generalized linear mixed model was fitted to the response "number of CFU", which was modeled to follow a negative binomial distribution. This distribution was used to accommodate the proportion of zero CFU observed. The statistical model used for analysis included the fixed effects of pressure differential (0 and -3.78 psi), hole size (10 and 100 μ m), presence of a carton (1=yes, 2 = no), and the 2-way interactions between pressure differential and carton, and between hole size and carton. The remaining interaction terms, the 2-way interaction between pressure differential and hole size and the 3-way interaction between all three effects, were considered for inclusion in the model but were dropped due to convergence problems. The model also included the random effect of chamber nested within pressure differential in order to recognize the experimental unit for this factor and the split plot arrangement of the experimental design. The model was fitted using the GLIMMIX procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC). Estimated number of CFU for combinations of the factors of interest, as well as differences between factor levels, were reported. Pairwise comparisons were conducted using Tukey-Kramer adjustment to avoid inflation of Type I error rate due multiple comparisons.

The null and research hypotheses for this analysis are:

- H₀: There will be no difference in the number of CFU between any of the test configurations.
- H_a: Trays containing a 10 µm hole, in the presence of a carton, and in the absence of a pressure differential will have the lowest number of CFU while trays containing a 100 µm hole, in the absence of a carton, and in the presence of a pressure differential will have the highest number of CFU.

The data did not support any 2-way interactions between the factors of interest. Similar patterns of effects were identified as those described for Question #1. Main effects of pressure differential (p< .0001), hole size (p<.0001), and secondary packaging (p=.0006) were identified.

5.4 Conclusions

The split plot design did not indicate any two- or three-way interactions between hole size, pressure differential, or secondary packaging, but it did indicate significant effects of each main factor. The sample size used was for 80% power; therefore, an

increased sample size would increase the level of power for the experimental design when investigating interaction effects for predicted probability of microbial ingress and number of CFU as response variables.

Hole size was identified, with significance (p<.0001), as a main effect when predicted probability of microbial ingress and number of CFU were response variables. It is intuitive that there is a relationship between hole size and microbe size when investigating how microorganisms travel through a hole defect. These results suggest that the larger the hole is, the easier it will be for a microorganism to travel through it. These results also indicate that it is possible for microorganism to travel through defects as small as 10 μ m, which is supportive of the continued need for integrity tests with high levels of sensitivity until modeling of the probability of contamination with multiple variables can be achieved.

Pressure differential was identified, with significance (p<.0001), as a main effect when predicted probability of microbial ingress and number of CFU were response variables. The levels of pressure differential used (0 and -3.78 psi) were outer boundaries of what could be anticipated in a distribution environment; the level of -3.78 psi is a worst case extreme during transit resulting from air craft or vehicle descent from 8,000 feet. Typically, devices are sterilized within their shipping containers, which provide additional layers of protection; therefore, the primary packages are not exposed to microorganisms within a typical distribution environment. However, pressure differentials that occur within in-use environments that are rich with microorganisms (healthcare facilities) are unknown. While these results identify that pressure

differential does serve as a driving force for microbes to travel through pinhole defects, more research is needed to determine what pressure differentials occur within hospitals (handling, doors opening and closing, etc.) and if those levels facilitate microbial penetration.

The presence or absence of a carton was identified, with significance, as a main effect when predicted probability of microbial ingress (p=.0146) and number of CFU (p=.0006) were response variables. It is intuitive that a carton will provide some level of barrier to microorganisms, but it also understood that it is not intended to serve as a sterile barrier. Significance was also identified when comparing secondary package type (pouches and cartons) when microbial penetration (p=.0102) and the number of CFU (p=.0279) were response variables. Here, pouches were identified as a better sterile barrier, as they did not have any microbial ingress. This supports industry practice, as pouches are secondary packages that are intended to serve as a sterile barrier. However, there are advantages to using cartons as a secondary package, as they provide label billboard, stacking stability in storage, and protection from distribution hazards. Trays which are packaged in pouches often require a carton as well, which adds a third layer of protective packaging, and also drive up costs as a result. In practice, the layer in contact with the device is intended as the sterile barrier; therefore trays packaged inside cartons have the ability to deliver a sterile device with the advantages of billboard, stackability, and protection while using a reduced amount of materials layers compared to trays packed in pouches.

5.5 Limitations

The following are identified as limitations for this research:

- Sample size the sample size used was calculated at an 80% power; there is potential to increase the level of power by increasing the sample size.
- Level of pressure differential the -3.78 psi pressure differential is typical of a distribution environment, but not necessarily an in-use environment.
- Microbial concentration the 1 x 10⁶ cells/ml concentration that was used is higher than what is representative of an in-use environment.

5.6 Future Research

The following are suggestions for future research:

- Increase the sample size it is possible that ingress could occur in trays with 10 μm holes without pressure differential, but the sample size was not large enough.
- Add levels to each factor (hole size and pressure differential) more data is
 required to understand the relationship between hole size and ingress (such as if
 it is a linear effect), which is necessary to develop a predictive model.
 Additionally, pressure differentials that occur during handling within the hospital
 environment and how those levels affect microbial ingress are unknown.
- Utilize the data for each factor to develop a mathematical model for the probability of contamination.

PINHOLE CHARACTERIZATION

When performing package integrity testing, in this case microbial challenge testing, it is necessary to create hole defects that are repeatable, consistent, and accurate; however, this can be challenging, particularly within the microcosm. Previous research has shown that excimer laser drilling, described as being one of the most precise and accurate techniques, results in holes with differing diameters at the entry and exit sides of the hole tunnels (Bix, Kassarjian et al. 2005). This raises questions as to what the shape and size are of the entire hole tunnel, and potential effects laser setup may have on microbial challenge testing.

6.1 Objectives

Study objectives were:

- to develop a methodology capable of objectively characterizing defects in PETG trays,
- and to use the newly developed methodology to characterize and compare thermal and excimer laser drilled holes in PETG trays.

6.2 Materials and Methods

While capturing images of the entry and exit sides of the holes on the packaging surfaces has been successful, capturing images of the entire hole tunnels has proven challenging. Here, methods were developed to obtain images of the entire hole tunnel using confocal microscopy.
A confocal laser scanning microscope was utilized due to its ability to capture high resolution fluorescence images, obtain stacks of images through the z-axis, and render those images three-dimensionally. These microscopes function by focusing a laser at one point on the sample; the excitation laser, focal point of the sample, and confocal aperture at the detector are all in conjugate focal locations. An objective directs in-focus light from the sample through the confocal aperture to the photomultiplier tube detector (PMT). The confocal aperture prevents out-of-focus light from reaching the PMT, resulting in optical sectioning (Figure 57). Confocal imaging provides the ability to look at layers of the sample along its z-axis, creating a stack of two-dimensional images called optical sections. Accompanying software can then be used to create three-dimensional renderings from the series of optical sections.



Figure 57. Confocal light pathways (Olympus 2011).

An Olympus FluoView FV1000 Confocal Laser Scanning Microscope and Olympus

FluoView BSW v. 5.0 software (Center Valley, PA) were used to obtain images of thermal

and excimer laser drilled holes in the trays. The holes were 100 μ m and 50 μ m in diameter, respectively. To prepare each sample, a Nikon SM2645 stereomicroscope was first used to identify the location of the hole on the tray. Then, the portion of the tray containing the hole was cut out so that it measured approximately 1.5 cm x 3 cm and marked with the tray identification number.

The samples required a coating to fill the hole tunnel (or coat the side walls) so that the light could interact with it to create an image. Initially, the samples were coated with osmium using an NEOC-AT osmium coater (Meiwafosis Co., Ltd., Osaka, Japan). This technique, however, was unsuccessful in providing a sufficient coating for reflected light interaction and image capture. While the osmium coated the surface of the tray and the side walls of the hole near the entry and exit sides, it did not penetrate the entire hole (Figure 58).



Figure 58. Osmium coating of an excimer laser drilled hole.

Instead, an aqueous solution of acridine orange dye (0.001%) was used to fill the holes in the samples so that the fluorescence could be used to image the tunnels. Nunc Lab-Tek II #1.5 Coverglass Chambers were used to hold the samples (Figure 50). First, a drop of acridine orange dye was added to the inside of the chamber using a pipette. Then the sample was placed inside the chamber on top of the dye so that the exterior side of the tray/sample was facing down (Figure 59). Lastly, an additional drop of acridine orange was placed on top of the sample at the previously identified location of the hole.



Figure 59. Sample inside a Lab-Tek II #1.5 Coverglass Chamber.

The microscope was configured with a stage plate designed to hold the Lab-Tek Chamber. The chamber was loaded onto the stage plate. Images were recorded using a UPlan FLN 20x (0.50) dry objective. The acridine orange fluorescence was detected through a 505 nm long pass emission filter following excitation by a 488 nm line of the Argon laser. Brightfield images were also simultaneously recorded on a separate PMT detector using the same 488nm Argon laser line (Figure 60).



Figure 60. Screen capture of imaging parameters.

Brightness and contrast for the fluorescent and Brightfield images were

optimized by adjusting PMT voltage and offset settings (Figures 61 and 62).



Figure 61. Figure 62. Thermal (Figure 61) and excimer (Figure 62) entry side laser hole images: fluorescent (top left), Brightfield (top right), and overlay of both (bottom).

Images were collected through the sample thickness with a z-step size of 10 μ m.

The thermal laser hole series was 390 μm and the excimer laser hole series was 380 μm

(Figures 63 and 64).



Figure 63. Thermal laser hole z-series images shown in stepwise progression.



Figure 64. Excimer laser hole z-series images shown in stepwise progression.

6.3 Results and Discussion

Three-dimensional renderings of the laser drilled hole tunnels were created as animations from the z-series images. The renderings displayed the side views of the tunnels, enabling a three-dimensional view of the defect in its entirety, so that their shapes and diameters could be identified (Figures 65 and 66). The acridine orange dye was successful in filling the hole tunnels and fluorescing; however, the exit sides of the holes appear dimmer than the entry sides. This is due to light scatter which limits the distance that the laser can travel through the sample. Light scatter occurs as the laser travels farther into the sample and as the sample emits the light back to the PMT. Gamma settings were adjusted to increase displayed color intensity so that the dimmer areas were more visible and the hole tunnel shape was more defined. Neither the thermal or excimer laser drilled holes exhibited precisely cylindrical shapes; the diameters within hole tunnels varied, with the entry sides having the largest diameters (Figures 65 and 66).



Figure 65. Figure 66. Side views of thermal (Figure 65) and excimer (Figure 66) laser hole 3D renderings.

Additionally, the entry sides of the holes were larger than they were certified to be (proclaimed drilling sizes). The thermal and excimer laser holes were drilled as 100 μ m and 50 μ m, respectively; however, their entry sides were much larger than those measurements in diameter (Figures 67 and 68). This is consistent with previous work conducted by the research team using two-dimensional views of the entry and exit sides of the holes employing a variety of microscopy techniques (Bix, Kassarjian et al. 2005). To provide an estimate of diameter for each hole type utilizing this methodology, measurements were taken from each hole and averaged to determine a mean hole size for the entry sides (Figures 67 and 68). The mean diameters were 229.96 µm for the thermal hole and 73.47 µm for the excimer hole (Table 8).



Figure 67. Figure 68. Entry side images and diameters of thermal (Figure 67) and excimer (Figure 68) laser drilled holes.

	Thermal	Excimer
Short diameter	216.12 μm	59.97 μm
Long diameter	243.8 μm	83.54 μm
Middle diameter	n/a	76.91 μm
Mean diameter	229.96 μm	73.47 μm

As seen with the 3D renderings, the entry sides of the tunnels are more clearly visible than the exit sides (Figures 65 and 66). In an effort to further improve these images, the following techniques can be refined:

- increase the objective from the UPlan FLN 20x (0.50) to a PlanN 40x (0.65)
- use a smaller z-step size increment (less than 10 μm)
- obtain a z-series from each side of the sample (both entry and exit sides).

The 40x objective will provide a higher resolution image because it has a better numerical aperture. The smaller z-step size will increase the number of optical sections and eliminate data gaps between slices along the hole tunnel. A z-series from each side of the sample will provide clearer imaging of both the entry and exit sides of the hole tunnel. Diameter measurements can then be more accurately obtained from the improved images so that described versus measured hole sizes can be compared for each hole type. Additionally, the 3D renderings of the hole tunnel shape will be more defined, resulting in a more descript comparison of thermal versus excimer hole tunnel shapes. APPENDICES

APPENDIX A

MATERIALS LIST

Trays

- Perfecseal, Mankato, MN
- Medtronic Inc. Outer Tray Part No. 350215-001
- Part material 0.025 inch blue tint uncoated polyethylene terephthalate (PETG)

Lidstock

- Amcor Flexibles Healthcare, Madison, WI
- LKF-002 Paper/PE/Foil/PE/HSC Die Cut Lids

Corrugated shippers

PE bags with twist ties

Tray Heat Sealer

- SenCorp, Hyannis, MA
- CeraTek model MD-2420 dual-shuttle heat sealer with tray 9-up tooling
- Teflon impregnated fiber glass barrier blanket

Holes

- Lenox Laser, Glen Arm, MD
- Laser drilled 10 μm (+/- 10%) and 100 μm (+/- 10%) holes in bottom of PETG trays
- Flow calibrated and labeled with tray ID, flow rate, and hole diameter

Non-destructive Leak Detector

- ATC, Inc., Indianapolis, IN
- Model VE2 with Leak-Tek[©] data acquisition program

Gamma Sterilization

- Provided by Smith & Nephew, Memphis, TN
- Performed by Sterigenics (West Memphis, AR) according to process procedure P000257 Rev. M.
- Shipping/sterilization cases
- SBS cartons for trays and pouches (protection for distribution)
- Kraft paper to fill gaps in cases

Media Preparation

- Media Prep Lab, Michigan State University, East Lansing, MI
- 30 ml of sterile nutrient growth agar in glass bottles sealed with foam stoppers
- Autoclave bin

• Insulated box constructed from EPS and corrugated

Agar Injection

- Multiterge
- Water bath
- Injection frame
- 70% isopropyl alcohol swabs
- 60 ml syringes (Becton, Dickinson and Company, Franklin Lakes, NJ)
- 18G1½ needles (Becton, Dickinson and Company, Franklin Lakes, NJ)
- 16G1 vented needles (Becton, Dickinson and Company, Franklin Lakes, NJ)
- Self-sealing septums (Mocon, Minneapolis, MN or Illinois Instruments, Johnsburg, IL)
- 70% isopropyl alcohol swabs
- Boats
- Bunsen burner
- Forceps

Butterfield's Solution

- Potassium Phosphate, Monobasic, Crystal (KH₂PO₄)
- 1N Sodium Hydroxide (1N NaOH)
- Latex gloves
- Pipette
- Distilled water
- Spatula
- Boats
- Scale (g)
- 2 L bucket
- Stir bar
- Corning Stirrer
- pH meter
- pH strips
- (4) 1 L Erlenmeyer flasks
- Autoclave bin
- Aluminum foil
- Autoclave tape
- Autoclave

Inoculation of Butterfield's Solution (Microbial Solution)

- Escherichia coli K12 ATCC Number 29181
- Pipettes (1 ml)
- Sterile pipette tips

Incubation

• Environmental chamber

Colony Counting of Test Samples

- Razor blade
- Sharpie

Colony Counting of Agar Plates

- Quebec Colony Counter
- Sharpie

Enterotubes

- BD BBL[™] Enterotube[™] II REF 211832
- Environmental chamber
- 1 ml syringe
- Kovac's reagent
- Latex gloves

Pouches

- Mangar Industries, Inc., New Britain, PA
- 100GA Biax Nylon (0.001), 0.0007 LDPE, 0.002 HDPE Coex
- Dimensions: 7.25 in x 9.50 in
- Lot #: H150978/1/A

Pouch Heat Sealer

- SenCorp, Hyannis, MA
- CeraTek Model No. 24AS/1, Serial No. 06-04236

Carton Construction

- 15.5 pt. SBS
- Glue
- Rubber bands
- Artios Kongsberg Premium Line 1930

Sample Preparation of Trays in Cartons

- Trays
- Cartons
- Self-sealing septums
- Template for septum alignment

Agar Preparation for Dilution Series Agar Plates

- Petri plates
- Difco[™] Nutrient Agar
- Deionized water
- Spatula
- Boats

- Scale (g)
- 500 ml Erlenmeyer flask
- Autoclave bin
- Aluminum foil
- Autoclave tape
- Autoclave
- Gloves

Dilution Series

- 3 dilution bottles
- 1 ml Escherichia coli K12 ATCC Number 29181
- Butterfield's Solution (99 ml in each dilution bottle)
- Wire autoclave basket
- Autoclave bin
- Autoclave tape
- Autoclave
- Pipettes (1 ml and 0.1 ml)
- Sterile pipette tips
- Boat
- 70% isopropyl alcohol
- Hockey stick
- Bunsen burner
- 6 sterile agar plates

Microbial Challenge

- Microbial Challenge Chamber
- Bleach
- Wash cloth
- 70% isopropyl alcohol swabs
- 60 ml syringes
- 18G1½ needles (Becton, Dickinson and Company, Franklin Lakes, NJ)
- Petri plates with sterile agar

Pinhole Characterization

- Olympus FluoView FV1000 Confocal Laser Scanning Microscope and Olympus FluoView BSW v. 5.0 software
- Nikon SM2645 stereomicroscope
- Nunc Lab-Tek II #1.5 Coverglass Chambers
- Scissors
- Sharpie
- Pipette
- Acridine orange dye 0.001% concentration
- Latex gloves

APPENDIX B

NON-DESTRUCTIVE LEAK TEST RESULTS

ID	Pass or Fail	ID	Pass or Fail	ID	Pass or Fail
1000	Passed	1036	Passed	1066	Passed
1001	Passed	1037	Passed	1067	Passed
1004	Failed	1038	Passed	1068	Passed
1006	Passed	1039	Passed	1069	Passed
1007	Passed	1041	Passed	1070	Passed
1008	Passed	1042	Passed	1071	Passed
1009	Passed	1043	Passed	1076	Passed
1011	Passed	1044	Passed	1077	Passed
1012	Passed	1045	Passed	1078	Passed
1013	Passed	1047	Passed	1079	Passed
1014	Passed	1048	Passed	1080	Passed
1015	Passed	1050	Passed	1081	Passed
1016	Passed	1051	Passed	1082	Passed
1017	Passed	1052	Passed	1083	Passed
1018	Passed	1053	Passed	1084	Passed
1019	Passed	1054	Passed	1085	Passed
1020	Passed	1055	Passed	1086	Passed
1023	Passed	1056	Passed	1087	Passed
1024	Passed	1057	Passed	1088	Passed
1025	Passed	1058	Passed	1089	Passed
1026	Passed	1059	Passed	1090	Passed
1027	Passed	1060	Passed	1091	Passed
1029	Passed	1061	Passed	1092	Passed
1030	Passed	1062	Passed	1093	Passed
1031	Passed	1063	Passed	1094	Passed
1032	Passed	1064	Passed	1095	Passed
1033	Passed	1065	Passed		

 Table 9. Non-destructive leak test results for trays without pinholes.

ID	Pass or Fail	ID	Pass or Fail	ID	Pass or Fail
1	Failed	68	Failed	130	Failed
2	Failed	69	Failed	131	Failed
3	Failed	71	Failed	132	Failed
7	Failed	73	Failed	165	Failed
8	Failed	74	Failed	166	Failed
9	Failed	75	Failed	167	Failed
10	Failed	76	Failed	168	Failed
11	Failed	77	Failed	169	Failed
12	Failed	78	Failed	170	Failed
13	Failed	79	Failed	171	Failed
14	Failed	81	Failed	172	Failed
15	Failed	82	Failed	173	Failed
16	Failed	83	Failed	174	Failed
17	Failed	84	Failed	175	Failed
18	Failed	85	Failed	176	Failed
19	Failed	86	Failed	177	Failed
20	Failed	87	Failed	178	Failed
21	Failed	88	Failed	179	Failed
22	Failed	89	Failed	180	Failed
23	Failed	90	Failed	181	Failed
24	Failed	91	Failed	182	Failed
25	Failed	92	Failed	183	Failed
27	Failed	93	Failed	184	Failed
28	Failed	94	Failed	185	Failed
29	Failed	95	Failed	186	Failed
30	Failed	96	Failed	187	Failed
31	Failed	97	Failed	188	Failed
32	Failed	98	Failed	189	Failed
33	Failed	99	Failed	190	Failed
35	Failed	100	Failed	191	Failed
36	Failed	101	Failed	192	Failed
37	Failed	102	Failed	193	Failed
38	Failed	103	Failed	194	Failed
39	Failed	104	Failed	195	Failed
40	Failed	105	Failed	196	Failed
41	Failed	106	Failed	197	Failed

Table 10. Non-destructive leak test results for trays with 10 μm pinholes.

Table 10 (cont'd).

-	-				
42	Failed	107	Failed	198	Failed
43	Failed	108	Failed	199	Failed
44	Failed	109	Failed	200	Passed
45	Failed	110	Failed	201	Failed
46	Failed	111	Failed	202	Failed
47	Failed	112	Failed	203	Failed
48	Failed	113	Failed	204	Failed
49	Failed	114	Failed	205	Failed
50	Failed	115	Failed	206	Failed
52	Failed	116	Failed	207	Failed
53	Failed	117	Failed	208	Failed
54	Failed	118	Failed	209	Failed
55	Failed	119	Failed	210	Failed
56	Failed	120	Failed	211	Failed
57	Failed	121	Failed	212	Failed
58	Failed	122	Failed	213	Failed
59	Failed	123	Failed	214	Failed
60	Failed	124	Failed	215	Failed
61	Failed	125	Failed	216	Failed
62	Failed	126	Failed	217	Failed
63	Failed	127	Failed	218	Failed
64	Failed	128	Failed	219	Failed
65	Failed	129	Failed	220	Failed

ID	Pass or Fail	ID	Pass or Fail	ID	Pass or Fail
2	Failed	96	Failed	200	Failed
3	Failed	97	Failed	201	Failed
4	Failed	98	Failed	202	Failed
5	Failed	99	Failed	205	Failed
6	Failed	100	Failed	206	Failed
7	Failed	101	Failed	207	Failed
8	Failed	102	Failed	208	Failed
9	Failed	103	Failed	209	Failed
10	Failed	104	Failed	210	Failed
11	Failed	105	Failed	212	Failed
12	Failed	106	Failed	213	Failed
13	Failed	107	Failed	214	Failed
14	Failed	108	Failed	215	Failed
15	Failed	109	Failed	216	Failed
16	Failed	110	Failed	217	Failed
17	Failed	111	Failed	218	Failed
18	Failed	112	Failed	219	Failed
19	Failed	113	Failed	220	Failed
20	Failed	117	Failed	221	Failed
21	Failed	118	Failed	222	Failed
22	Failed	119	Failed	223	Failed
23	Failed	120	Failed	224	Failed
24	Failed	121	Failed	225	Failed
25	Failed	122	Failed	226	Failed
26	Failed	123	Failed	227	Failed
27	Failed	124	Failed	228	Failed
28	Failed	125	Failed	229	Failed
29	Failed	126	Failed	230	Failed
30	Failed	127	Failed	231	Failed
31	Failed	128	Failed	232	Failed
32	Failed	129	Failed	233	Failed
33	Failed	130	Failed	234	Failed
34	Failed	131	Failed	235	Failed
35	Failed	132	Failed	236	Failed
36	Failed	133	Failed	237	Failed
37	Failed	134	Failed	238	Failed
38	Failed	136	Failed	239	Failed

Table 11. Non-destructive leak test results for trays with 100 μm pinholes.

Table 11 (cont'd).

39	Failed	139	Failed	240	Failed
40	Failed	143	Failed	241	Failed
41	Failed	144	Failed	242	Failed
42	Failed	145	Failed	243	Failed
43	Failed	146	Failed	244	Failed
44	Failed	147	Failed	245	Failed
45	Failed	148	Failed	246	Failed
46	Failed	149	Failed	247	Failed
47	Failed	150	Failed	248	Failed
48	Failed	151	Failed	249	Failed
49	Failed	152	Failed	250	Failed
50	Failed	153	Failed	251	Failed
54	Failed	154	Failed	252	Failed
55	Failed	155	Failed	253	Failed
56	Failed	156	Failed	254	Failed
57	Failed	157	Failed	255	Failed
58	Failed	158	Failed	256	Failed
59	Failed	159	Failed	257	Failed
60	Failed	160	Failed	258	Failed
61	Failed	161	Failed	261	Failed
62	Passed	162	Failed	262	Failed
63	Failed	164	Failed	263	Failed
64	Failed	165	Failed	264	Failed
65	Failed	166	Failed	265	Failed
66	Failed	167	Failed	266	Failed
67	Failed	168	Failed	267	Failed
68	Failed	169	Failed	268	Failed
69	Failed	170	Failed	269	Failed
70	Failed	172	Failed	270	Failed
71	Failed	174	Failed	271	Failed
72	Failed	176	Failed	272	Failed
73	Failed	177	Failed	273	Failed
74	Failed	178	Failed	275	Failed
75	Failed	179	Failed	276	Failed
76	Failed	180	Failed	277	Failed
77	Failed	181	Failed	278	Failed
78	Failed	182	Failed	279	Failed
79	Failed	183	Failed	280	Failed

Table 11 (cont'd).

80	Failed	184	Failed	281	Failed
81	Failed	185	Failed	282	Failed
82	Failed	186	Failed	283	Failed
83	Failed	187	Failed	284	Failed
84	Failed	188	Failed	285	Failed
85	Failed	189	Failed	286	Failed
86	Failed	190	Failed	287	Failed
87	Failed	191	Failed	288	Failed
88	Failed	192	Failed	289	Failed
89	Failed	193	Failed	290	Failed
90	Failed	194	Failed	291	Failed
91	Failed	195	Failed	292	Failed
92	Failed	196	Failed	293	Failed
93	Failed	197	Failed	294	Failed
94	Failed	198	Failed	295	Failed
95	Failed	199	Failed	297	Failed

APPENDIX C

PART 1 TEST SAMPLE RESULTS

ID	Injection Run	Injection Position (A,B,C,D)	Chamber Run	Chamber Position (A,B,C,D)	Hole Size (µm)	Flow Rate (sccm)	Pressure (psi)	Count (CFU)
159	11	А	8	А	100	174.89	-3.78	69
164	12	А	11	D	99.41	172.85	-3.78	9
167	12	D	15	А	96.3	162.18	-3.78	11
168	13	С	9	D	96.73	163.66	-3.78	1
210	16	D	13	D	93.63	153.34	-3.78	11
212	11	С	12	С	98.73	170.49	-3.78	31
213	16	С	14	В	108	204.00	-3.78	15
221	15	D	10	С	95.95	161.02	-3.78	20
223	15	С	14	С	97.37	165.83	-3.78	7
224	15	В	15	D	99.82	174.27	-3.78	7
226	17	В	13	В	96.1	161.51	-3.78	10
262	12	В	11	А	101.69	180.87	-3.78	74
273	13	В	10	В	93.71	153.58	-3.78	2
278	13	А	9	В	105.64	195.19	-3.78	18
284	12	С	12	А	94.56	156.40	-3.78	31
136	18	А	15	В	103.8	188.44	0	2
160	15	А	11	С	101.85	181.44	0	0
165	13	D	14	D	92.77	150.53	0	2
200	17	С	12	В	100.72	177.44	0	3
207	17	D	12	D	93.59	153.19	0	0
208	18	С	15	С	93.34	152.37	0	2
218	10	D	9	С	100.15	175.43	0	0
225	16	А	8	В	95.3	158.85	0	0
227	17	А	14	А	98.56	169.91	0	1
263	11	В	10	А	91.93	147.80	0	0
266	11	D	10	D	99.23	172.23	0	1
269	16	В	11	В	105.37	194.18	0	1
279	14	В	8	D	104.65	191.56	0	3
280	14	А	9	А	103.23	186.37	0	0
282	14	D	13	С	106.21	197.28	0	1

 Table 12. Part 1 test sample colony counts (CFU).

APPENDIX D

PART 1 AGAR PLATE COLONY COUNTS

Chamber Run	Count (CFU)
1	4332
2	4284
3	4410
4	4568
5	4316
6	4111
7	3906
8	3946

Table 13. Part 1 microbial chamber agar plate colony counts (CFU per plate).

APPENDIX E

PART 2 AGAR PLATE COLONY COUNTS

Table 14. Microbial chamber agar plate colony counts (CFU per plate) for runs testing unlidded trays in pouches.

Chamber Run	Count (CFU)
1	7576
2	7907
3	5607
4	4631
5	3024
6	2016
7	1276
8	1055
9	1150
10	1134

Table 15. Microbial chamber agar plate colony counts (CFU per plate) for runs testing unlidded trays in cartons.

Chamber Run	Count (CFU)
1	6615
2	5654
3	6505
4	5843
5	4473
6	3827
7	3386
8	2615
9	2331
10	2142

Table 16. Microbial chamber agar plate colony counts (CFU per plate) for runs testing lidded trays, with 100 μ m pinholes, in cartons.

Chamber Run	Count (CFU)
1	9072
2	10584
3	7308
4	6867
5	6930
6	4662
7	3906
8	3024
9	3150
10	3528

APPENDIX F

PART 2 TEST SAMPLE RESULTS

ID	Injection Number	Chamber Run	Chamber Position (A,B,C,D)	Count (CFU)
2000	24	7	С	0
2001	37	6	D	0
2002	39	5	D	0
2003	40	5	С	0
2004	30	8	С	0
2005	31	7	А	0
2006	32	7	В	0
2007	17	9	D	0
2008	3	2	В	0
2010	5	1	С	0
2011	14	3	В	0
2012	4	1	D	0
2013	26	10	С	0
2014	25	10	D	0
2015	34	6	А	0
2016	33	6	В	0
2017	23	7	D	0
2018	2	2	С	0
2020	12	3	D	0
2021	22	8	A	0
2023	7	1	A	0
2024	41	5	A	0
2026	6	1	В	0
2028	11	4	А	0
2029	1	2	D	0
2030	18	9	С	0
2031	28	10	А	0
2033	38	6	С	0
2034	19	9	В	0
2035	20	9	A	0
2036	9	4	С	0
2037	10	4	В	0
2038	13	3	С	0
2039	21	8	В	0

Table 17. Colony counts (CFU) of unlidded trays in pouches.

Table 17 (cont'd).

2043	29	8	D	0
2044	8	2	А	0
2045	16	4	D	0
2046	15	3	А	0
2049	27	10	В	0

Table 18. Colony counts (CFU) of unlidded trays in cartons.

ID	Injection Number	Chamber Run	Chamber Position (A,B,C,D)	Count (CFU)
3000	1	10	D	0
3001	9	9	Α	2
3002	8	7	А	10
3005	37	1	А	3
3006	38	2	В	2
3007	30	5	А	124
3008	31	2	D	62
3009	32	1	В	15
3010	40	3	С	88
3012	5	10	С	0
3016	22	4	С	19
3017	2	9	С	1
3018	19	7	В	7
3020	6	9	В	5
3021	33	4	А	8
3024	12	6	А	4
3025	13	10	А	21
3026	14	8	D	65
3027	15	7	С	3
3028	16	6	D	15
3029	17	9	D	3
3030	18	8	С	2
3031	27	3	А	12
3032	10	10	В	3
3033	21	5	С	7
3034	35	2	С	10
3035	3	8	Α	1
3036	7	8	В	2
3037	23	3	В	41
3042	4	6	В	3

Table 18 (cont'd).

3044	11	7	D	4
3045	39	4	D	2
3046	26	5	В	49
3048	20	6	С	2
3049	34	5	D	5
3050	29	3	D	101
3051	28	1	С	4
3052	24	2	А	8
3053	25	4	В	40

Table 19. Colony counts (CFU) of lidded trays, with 100 μm pinholes, in cartons.

ID	Injection Number	Chamber Run	Chamber Position (A, B, C, D)	Hole Size (µm)	Flow Rate (sccm)	Count (CFU)
66	35	2	В	101.82	181.33	0
69	33	3	В	92.14	148.49	0
71	38	1	В	93.34	152.39	0
92	25	7	D	98.85	170.91	0
93	32	3	А	98.82	170.80	0
94	29	3	С	97.77	167.18	0
95	30	4	С	106.5	198.51	0
152	14	8	D	98.66	170.25	0
153	24	9	А	93.51	152.93	0
154	34	2	С	93.15	151.75	0
187	7	7	В	97.12	164.98	0
188	11	9	D	101.46	180.04	0
189	12	10	С	96.61	163.25	0
191	17	3	D	91.57	146.65	0
192	10	4	А	101.11	178.81	0
194	16	5	С	99.69	173.83	0
197	28	4	В	94.54	156.31	1
228	26	6	В	108.11	204.41	0
231	31	2	D	97.95	167.80	0
240	40	1	А	94.67	156.75	0
245	5	8	С	102.39	183.36	0
246	1	10	В	93.14	151.73	1
247	20	7	A	99.73	173.97	1
248	2	9	В	92.67	150.20	0
249	19	5	A	95.94	160.99	0
251	37	1	С	100.28	175.87	0

Table 19 (cont'd).

	-					
252	18	8	А	104.68	191.66	0
254	39	1	D	90.04	141.78	0
255	15	8	В	91.15	145.33	0
256	13	9	С	98.64	170.16	1
257	3	7	С	101.9	181.61	0
287	36	2	А	101.14	178.92	0
288	9	5	D	103.65	187.92	0
289	22	4	D	106.35	197.80	0
290	4	6	С	103.16	186.12	1
291	8	6	D	104.68	191.67	0
292	23	10	D	106.52	198.47	0
293	6	10	А	102.82	184.91	0
294	21	6	A	102.53	183.88	0
295	27	5	В	104.68	191.64	1

APPENDIX G

PART 3 AGAR PLATE COLONY COUNTS

Chamber	Count	Chamber	Count	Chamber	Count
Run	(CFU)	Run	(CFU)	Run	(CFU)
1	26649	26	23247	51	19278
2	23814	27	20412	52	23247
3	22113	28	22680	53	19278
4	24381	29	19845	54	17577
5	23247	30	16443	55	24381
6	20412	31	19278	56	23247
7	23247	32	21546	57	24948
8	22680	33	19845	58	24381
9	17577	34	17010	59	22113
10	15309	35	19845	60	21546
11	22113	36	18711	61	21546
12	21546	37	17577	62	22113
13	24948	38	20412	63	22680
14	24381	39	17577	64	21546
15	23814	40	15876	65	18144
16	21546	41	11907	66	19278
17	20412	42	13608	67	24948
18	19278	43	23247	68	24381
19	21546	44	23814	69	25515
20	18711	45	20979	70	26082
21	17577	46	23814	71	26649
22	20412	47	20412	72	25515
23	18144	48	23814	73	21546
24	20979	49	22113	74	23247
25	24381	50	19845	75	24381
				76	22113

 Table 20. Part 3 microbial chamber agar plate colony counts (CFU per plate).

APPENDIX H

PART 3 TEST SAMPLE RESULTS

ID	Chamber Run	Chamber Position (A, B, C, D)	Hole Size (µm)	Flow Rate (sccm)	Carton (1=yes, 2=no)	Pressure (psi)	Count (CFU)
229	1	A	104.72	191.784	1	-3.78	1
81	1	В	10.18	1.812	2	-3.78	0
52	1	С	8.28	1.2	1	-3.78	0
20	1	D	103.99	189.128	2	-3.78	32
176	2	А	91.06	145.034	2	0	0
70	2	В	91	144.846	1	0	0
165	2	С	10.77	2.03	2	0	0
63	2	D	9.15	1.464	1	0	0
190	3	А	10.79	2.035	1	-3.78	0
125	3	В	94.42	155.932	2	-3.78	74
100	3	С	104.82	192.163	1	-3.78	2
92	3	D	9.53	1.589	2	-3.78	0
150	4	А	102.88	185.111	1	0	1
33	4	В	102.08	182.262	2	0	0
203	4	С	11.46	2.297	2	0	0
124	4	D	11.11	2.158	1	0	0
42	5	А	11.96	2.5	2	-3.78	0
149	5	В	107.69	202.825	1	-3.78	4
80	5	С	102.82	184.911	2	-3.78	132
208	5	D	11.84	2.453	1	-3.78	0
121	6	А	99.29	172.426	2	0	0
108	6	В	8.25	1.19	1	0	0
110	6	С	8.31	1.207	2	0	0
230	6	D	100.24	175.755	1	0	0
213	7	А	9.19	1.477	2	-3.78	0
31	7	В	11.37	2.263	1	-3.78	0
39	7	С	107.61	202.521	1	-3.78	3
84	7	D	96.89	164.194	2	-3.78	148
18	8	А	101.87	181.5	2	0	0
97	8	В	8	1.12	2	0	0
35	8	С	10.35	1.875	1	0	0

 Table 21. Colony counts (CFU) of split plot test samples.

Table 21 (cont'd).

	• •						
42	8	D	96.99	164.539	1	0	0
109	9	А	8.25	1.19	2	-3.78	0
122	9	В	93.92	154.268	2	-3.78	33
32	9	С	9.09	1.446	1	-3.78	0
47	9	D	107.97	203.898	1	-3.78	16
28	10	А	9.79	1.678	1	0	0
195	10	В	94.4	155.861	1	0	0
124	10	С	102.16	182.528	2	0	3
194	10	D	11.01	2.121	2	0	0
43	11	A	93.26	152.109	1	-3.78	1
173	11	В	11.14	2.17	2	-3.78	0
130	11	С	106.26	197.471	2	-3.78	72
47	11	D	10.64	1.98	1	-3.78	0
166	12	А	9.17	1.472	2	0	0
193	12	В	95.73	160.268	1	0	0
116	12	С	9.17	1.472	1	0	0
264	12	D	97.13	164.992	2	0	1
123	13	А	8	1.12	1	-3.78	0
190	13	В	101.31	179.51	1	-3.78	3
183	13	С	95.4	159.182	2	-3.78	152
207	13	D	10.43	1.902	2	-3.78	0
82	14	A	97.98	167.901	2	0	0
94	14	В	10.78	2.031	2	0	0
40	14	С	107.09	200.577	1	0	0
113	14	D	8.25	1.19	1	0	0
20	15	А	10.33	1.868	2	-3.78	0
40	15	В	11	2.117	1	-3.78	0
184	15	С	93.17	151.81	2	-3.78	146
45	15	D	99.72	173.932	1	-3.78	0
120	16	А	10.02	1.755	1	0	0
81	16	В	98.58	169.966	2	0	3
18	16	С	10.07	1.773	2	0	0
198	16	D	96.92	164.293	1	0	0
133	17	А	97.07	164.794	2	-3.78	86
37	17	В	9.25	1.496	1	-3.78	0
237	17	С	97.14	165.028	1	-3.78	0
191	17	D	10.82	2.047	2	-3.78	0
233	18	A	104.22	189.963	1	0	0

Table 21 (cont'd).

	• •						
83	18	В	98.39	169.307	2	0	0
107	18	С	10.13	1.796	1	0	0
193	18	D	11.87	2.462	2	0	0
196	19	A	91.39	146.089	1	-3.78	0
121	19	В	11.54	2.33	1	-3.78	0
104	19	С	8.25	1.19	2	-3.78	2
16	19	D	99.97	174.789	2	-3.78	41
127	20	А	98.32	169.06	2	0	4
232	20	В	96.19	161.82	1	0	0
211	20	С	10.79	2.037	1	0	0
192	20	D	10.57	1.1954	2	0	0
117	21	А	11.74	2.41	1	-3.78	0
100	21	В	11.86	2.46	2	-3.78	0
110	21	С	99.63	173.624	1	-3.78	0
123	21	D	99.43	172.9	2	-3.78	15
12	22	А	9.23	1.49	2	0	0
28	22	В	97.45	166.11	2	0	1
32	22	С	104.19	189.846	1	0	0
39	22	D	9	1.418	1	0	0
114	23	А	9.13	1.459	1	-3.78	0
195	23	В	10.64	1.98	2	-3.78	0
131	23	С	107.1	200.606	2	-3.78	171
234	23	D	103.75	188.272	1	-3.78	0
113	24	А	106.57	198.622	1	0	0
182	24	В	10.84	2.054	1	0	0
27	24	С	98.21	168.703	2	0	0
98	24	D	8.75	1.34	2	0	0
38	25	А	94.89	157.495	1	-3.78	3
10	25	В	8.68	1.318	2	-3.78	0
36	25	С	9.63	1.623	1	-3.78	0
87	25	D	105.7	195.39	2	-3.78	84
60	26	А	90.19	142.278	2	0	0
41	26	В	105.46	194.515	1	0	0
93	26	С	11.84	2.45	2	0	0
33	26	D	8.78	1.347	1	0	0
27	27	A	10.08	1.778	1	-3.78	0
30	27	В	100.12	175.323	2	-3.78	0
108	27	С	104.4	190.619	1	-3.78	1

Table 21 (cont'd).

	• •						
46	27	D	8.01	1.121	2	-3.78	0
109	28	А	106.98	200.177	1	0	0
132	28	В	107.31	201.416	2	0	0
45	28	С	9.66	1.631	2	0	0
185	28	D	9.75	1.661	1	0	0
44	29	А	8.1	1.148	2	-3.78	0
88	29	В	102.58	184.044	1	-3.78	1
120	29	С	97.15	165.059	2	-3.78	11
64	29	D	8.71	1.327	1	-3.78	0
29	30	А	93.11	151.613	2	0	3
188	30	В	10.72	2.009	1	0	0
43	30	С	9.53	1.589	2	0	0
31	30	D	97.98	167.905	1	0	0
14	31	Α	8.04	1.13	2	-3.78	0
29	31	В	8.39	1.23	1	-3.78	0
25	31	С	96.54	163.019	1	-3.78	2
134	31	D	107	200.226	2	-3.78	108
101	32	А	102.14	182.468	2	0	0
16	32	В	11.78	2.427	2	0	0
122	32	С	8	1.12	1	0	0
64	32	D	104.44	190.779	1	0	0
202	33	Α	9.28	1.507	2	-3.78	0
21	33	В	90.16	142.164	2	-3.78	63
62	33	С	11.02	2.124	1	-3.78	0
111	33	D	103.14	186.068	1	-3.78	0
61	34	А	8.82	1.36	1	0	0
74	34	В	105.62	195.13	1	0	0
22	34	С	90.35	142.785	2	0	0
2	34	D	11.25	2.213	2	0	0
105	35	А	107.75	203.063	1	-3.78	3
175	35	В	11.09	2.151	2	-3.78	0
98	35	С	104.26	190.105	2	-3.78	34
132	35	D	11.96	2.503	1	-3.78	0
95	36	А	11.96	2.5	2	0	0
26	36	В	95.07	158.093	1	0	0
180	36	С	11.9	2.477	1	0	0
90	36	D	99.66	173.719	2	0	0
115	37	А	9.03	1.427	1	-3.78	0

Table 21 (cont'd).

	• •						
236	37	В	98.03	168.085	1	-3.78	1
23	37	С	9.77	1.67	2	-3.78	0
86	37	D	105.86	196.013	2	-3.78	86
58	38	A	94.9	157.503	2	0	0
21	38	В	11.82	2.446	2	0	0
46	38	С	104.82	192.163	1	0	0
87	38	D	10.31	1.859	1	0	0
176	39	А	8.13	1.156	2	-3.78	0
38	39	В	8	1.12	1	-3.78	0
78	39	С	100.38	176.234	2	-3.78	61
235	39	D	106.25	197.447	1	-3.78	8
127	40	A	8	1.12	1	0	0
49	40	В	102.62	184.186	2	0	1
204	40	С	10.13	1.794	2	0	0
99	40	D	100.42	176.367	1	0	0
77	41	A	103.11	185.96	2	-3.78	1
184	41	В	8	1.12	1	-3.78	1
75	41	С	105.31	193.968	1	-3.78	1
219	41	D	10.58	1.957	2	-3.78	0
61	42	А	102.19	182.63	1	0	0
144	42	В	96.04	161.336	2	0	1
119	42	С	11.22	2.204	1	0	0
7	42	D	11.96	2.5	2	0	0
180	43	A	92.14	148.478	1	-3.78	2
58	43	В	10.43	1.904	1	-3.78	0
106	43	С	10.73	2.013	2	-3.78	0
179	43	D	91.78	147.331	2	-3.78	0
145	44	А	96.87	164.12	2	0	0
68	44	В	98.66	170.235	1	0	0
174	44	С	9.13	1.457	1	0	0
216	44	D	8.95	1.401	2	0	0
55	45	А	9.32	1.519	1	-3.78	0
170	45	В	10.11	1.788	2	-3.78	0
182	45	С	90.21	142.328	1	-3.78	4
178	45	D	103.95	188.986	2	-3.78	106
197	46	А	9.26	1.499	2	0	0
13	46	В	95.46	159.391	2	0	1
241	46	С	102.33	183.14	1	0	1

Table 21 (cont'd).

	• •						
50	46	D	10.67	1.99	1	0	0
181	47	А	11.95	2.497	1	-3.78	0
214	47	В	9.89	1.709	2	-3.78	4
12	47	С	91.42	146.183	2	-3.78	173
151	47	D	99.85	174.373	1	-3.78	1
36	48	А	101.64	180.672	1	0	0
169	48	В	11.4	2.271	1	0	0
148	48	С	92.65	150.126	2	0	0
15	48	D	10.78	2.034	2	0	0
35	49	А	102.09	182.277	1	-3.78	7
199	49	В	11.66	2.378	2	-3.78	0
130	49	С	11.01	2.12	1	-3.78	0
147	49	D	93.45	152.74	2	-3.78	97
181	50	А	97.59	166.58	2	0	0
63	50	В	99.62	173.588	1	0	0
26	50	С	10.03	1.76	2	0	0
74	50	D	10.09	1.78	1	0	0
15	51	А	94.31	155.552	2	-3.78	0
23	51	В	96.1	161.523	1	-3.78	0
205	51	С	9.23	1.488	2	-3.78	0
25	51	D	10.53	1.939	1	-3.78	0
24	52	А	92.45	149.498	1	0	0
174	52	В	94.82	157.236	2	0	0
168	52	С	11.03	2.127	2	0	0
56	52	D	10.66	1.988	1	0	0
83	53	А	10.32	1.863	2	-3.78	0
242	53	В	102.69	184.421	1	-3.78	15
14	53	С	101.47	180.096	2	-3.78	60
71	53	D	8.05	1.132	1	-3.78	0
146	54	А	99.2	172.105	2	0	2
178	54	В	11.72	2.404	1	0	0
13	54	С	10.13	1.796	2	0	0
112	54	D	104.31	190.3	1	0	0
103	55	А	8.25	1.19	2	-3.78	0
111	55	В	11.86	2.459	1	-3.78	0
106	55	С	99.37	172.709	1	-3.78	4
6	55	D	96.58	163.146	2	-3.78	42
129	56	A	94.65	156.674	2	0	0
Table 21 (cont'd).

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102	56	В	8.32	1.21	2	0	0
73	56	С	11.4	2.271	1	0	0
186	56	D	107.21	201.015	1	0	0
9	57	А	8	1.12	2	-3.78	0
79	57	В	97.5	166.251	2	-3.78	143
60	57	С	8.96	1.404	1	-3.78	0
102	57	D	105.16	193.423	1	-3.78	1
69	58	А	8.79	1.35	1	0	0
253	58	В	101.83	181.346	1	0	0
59	58	С	95.61	159.875	2	0	0
24	58	D	10.12	1.79	2	0	0
222	59	А	92.67	150.189	1	-3.78	0
212	59	В	8.34	1.215	2	-3.78	0
85	59	С	91.73	147.169	2	-3.78	115
118	59	D	9.46	1.567	1	-3.78	0
220	60	А	8.84	1.366	2	0	0
96	60	В	102.53	183.86	1	0	0
189	60	С	11.79	2.43	1	0	0
7	60	D	95.85	160.698	2	0	0
172	61	А	8	1.12	1	-3.78	0
104	61	В	107.26	201.204	1	-3.78	6
11	61	С	8.39	1.23	2	-3.78	0
4	61	D	103.04	185.693	2	-3.78	70
76	62	А	101.48	180.103	2	0	0
82	62	В	9.13	1.458	2	0	0
239	62	С	107.16	200.835	1	0	0
57	62	D	10.04	1.761	1	0	0
41	63	А	12	2.52	2	-3.78	0
131	63	В	9.41	1.55	1	-3.78	0
128	63	С	97.43	166.025	2	-3.78	232
243	63	D	97.66	166.813	1	-3.78	0
79	64	А	10.48	1.923	1	0	0
57	64	В	91.73	147.176	2	0	1
218	64	С	10.58	1.957	2	0	0
34	64	D	108.04	204.171	1	0	0
5	65	А	93.31	152.268	2	-3.78	2
68	65	В	9.97	1.74	1	-3.78	0
103	65	С	100.65	177.17	1	-3.78	2

Table 21 (cont'd).

	• •						
96	65	D	8.02	1.124	2	-3.78	0
185	66	А	102.64	184.251	1	0	0
17	66	В	103.71	188.121	2	0	6
77	66	С	9.13	1.458	1	0	0
177	66	D	9.29	1.509	2	0	0
244	67	А	99.51	173.194	1	-3.78	1
217	67	В	9.85	1.696	1	-3.78	0
19	67	С	10.07	1.773	2	-3.78	0
11	67	D	103.45	187.191	2	-3.78	9
8	68	А	92.37	149.216	2	0	0
238	68	В	105.26	193.767	1	0	0
48	68	С	10.15	1.803	1	0	0
89	68	D	8.35	1.22	2	0	0
53	69	А	11.71	2.398	1	-3.78	0
196	69	В	11.25	2.215	2	-3.78	0
250	69	С	106.7	199.116	1	-3.78	3
10	69	D	95.25	158.684	2	-3.78	54
167	70	А	9.1	1.45	2	0	0
50	70	В	98.05	168.16	2	0	0
97	70	С	106.06	196.722	1	0	0
75	70	D	11.15	2.174	1	0	0
65	71	А	11.43	2.286	1	-3.78	0
210	71	В	10.82	2.047	2	-3.78	0
91	71	С	101.58	180.454	2	-3.78	286
44	71	D	95.3	158.83	1	-3.78	2
56	72	А	103.44	187.126	1	0	0
3	72	В	11.24	2.208	1	0	0
48	72	С	101.3	179.48	2	0	1
91	72	D	8.42	1.24	2	0	0
54	73	А	105.05	193.004	1	-3.78	2
22	73	В	11.23	2.207	2	-3.78	0
171	73	С	11.87	2.464	1	-3.78	0
2	73	D	100.69	177.309	2	-3.78	7
177	74	А	104.19	189.877	2	0	0
19	74	В	96.36	162.4	1	0	0
17	74	С	8.09	1.144	2	0	0
76	74	D	10.61	1.967	1	0	0
30	75	A	8.96	1.404	1	-3.78	0

9	75	В	97.47	166.161	2	-3.78	36
65	75	С	102.39	183.37	1	-3.78	6
85	75	D	10.66	1.986	2	-3.78	1
126	76	А	98.01	168.011	1	0	0
55	76	В	90.87	144.427	2	0	1
112	76	С	8.25	1.19	2	0	0
198	76	D	9.61	1.617	1	0	0

Table 21 (cont'd).

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