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THE EFFECT OF PRESSURE DIFFERENTIAL ON MICROBIAL PENETRATION OF A STERILE MEDICAL DEVICE TRAY

By

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ABSTRACT

THE EFFECT OF PRESSURE DIFFERENTIAL ON MICROBIAL PENETRATION OF A STERILE MEDICAL DEVICE TRAY

By

Jane Erin Severin

A developmental need of the medical device industry is a body of knowledge regarding the required level of sensitivity for package integrity tests. As a result of this need, the objective of this research was twofold: (1) to create and verify a new methodology for whole-package microbial testing for medical device packages and (2) to examine the effect of pressure differential on microbial ingress in a sterile medical device tray.

The new method was a clear improvement over existing techniques because it reduced the chance of false positives that were commonly observed in the previous tests of integrity. The method involved aseptically filling sealed, sterile device packages with a known volume of an appropriate growth medium, exposing the packages to a microbial challenge, incubating the package and inspecting it for growth.

After creating and refining the test technique, further work explored the impact of pressure differential and a limited number of hole sizes (0, 10, and 100 micron) on microbial penetration. The specific pressure differential examined simulates the descent of an aircraft from 8000 feet or a ground shipment descending from 8000 feet. While air transportation is a common mode for medical device distribution, it is important to recognize that packages are routinely exposed to pressure differentials in all elements of handling.

Pressure differential and hole size had a statistically significant effect on the microbial penetration of the sterile medical device test tray used in this study. The critical penetration threshold is less than 100 microns. These results and the development and verification of a whole package microbial challenge test method provide useful information with which to build future research that seeks to determine the required level of sensitivity for packaging integrity tests for sterile packaging.

This Dissertation is dedicated to my husband, Paul, and our children, Brenna and Jack, in appreciation for their sacrifice, and for their love and support

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CHAPTER 1

INTRODUCTION

The Legislation and Regulation of Medical Devices

Medical devices range from simple items, like tongue depressors and bedpans, to complex pieces of equipment, such as programmable pacemakers with micro-chip technology. A device is defined by section 201(h) of the Federal Food, Drug, and Cosmetic Act (FD&C Act) as:

> "an instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent or other similar or related article, including a component part, or accessory which is:
>
> - in the official National Formulary, or the United States Pharmacopoeia, or any supplement to them,
> - intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals, or,
> - intended to affect the structure or any function of the body of man or other animals, and which does not achieve any of it's primary intended purposes through chemical action within or on the body of man or other animals and which is not dependent upon being metabolized for the achievement of any of its primary intended purposes." (U.S. Food and Drug Administration. Federal Food Drug and Cosmetic Act (FD&C), 1938).

If a product is labeled, promoted, or used in a manner that meets this definition, it will be regulated by the Food and Drug Administration (FDA) as a medical device. As such,

it is subject to regulatory control. The level of regulatory control is dependent on the device's inherent risk, and its intended use and indications for use (http://www.fda.gov/cdrh/devadvice/313.html, 2005).

The level of scrutiny employed by the FDA falls into three broad regulatory classes: Class I devices represent the lowest level of patient risk; these devices require only the general controls dictated by the FD&C Act of 1938. Classes II and III require not only the general controls, but also an enhanced level of regulatory scrutiny dictated by the 1976 Medical Device Amendment (Code of Federal Regulations, Title 21, Volume 8, Part 814-Pre-Market Approval of Medical Devices Amendment, 1976) (See Table 1) (Rosen, 2004).

Table 1- Classification of Medical Devices (Rosen, 2004)

Device Class	Level Of Control	Risk Level	% Of All Devices	Examples
Class I	General Controls	Low	46%	Dental Floss, Arm Slings
Class II	General Controls and Special Controls	Moderate	47%	Facial Implants, Catheters
Class III	General Controls and Premarket Approval	Moderate to High	7%	Pace Makers, Vascular Grafts

The State of the Industry

The medical device industry, while considerably smaller in sales dollars than its pharmaceutical counterpart, is a growth market. In 2004, sales were reported at $100 billion and grew at a 9 percent rate per year, while the pharmaceutical industry had annual sales of $500 billion and grew at a rate of 7 percent (Rosen, 2004; Trombetta, 2005). Growth has been the hallmark of many major segments of the medical device industry (Figure 1).

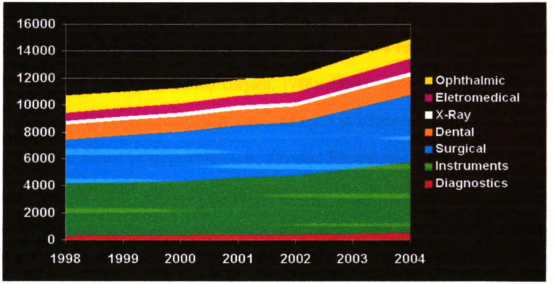


Figure 1 – Growth in Various Segments of the Medical Device Industry – Obtained From http://www.fda.gov/cdrh/present/fdli05/index_files/images/image56.png

The following are key factors driving the growth of the industry (Rosen, 2004):

1. 'The accelerated aging of the United States, European, and Japanese populations (particularly age groups over 65 years).'
2. 'The accelerated integration of drugs and devices.'
3. 'The interface of nanotechnology and medical device industry.' This facilitates the reduction in size of many devices.
4. 'The expanded use of diagnostics to customize therapeutic regimens on a patient level and to aid in the prediction of disease onset'.

Issues of Health and Package Integrity

Producing medical devices that maintain sterility until they reach the end-user is a persistent challenge for industry. According to figures compiled by the FDA, packaging accounted for 42.5 percent of sterility-related recalls in 1986, and 40 percent of sterility-related recalls in 1987 (Bryant, 1988). These figures prompted additional FDA scrutiny of manufacturing processes and resulted in the implementation of additional controls

(Food and Drug Administration, Center for Devices and Radiologic Health, QSR Manual, Section 820.70). While these measures have resulted in a reduction in the number of recalls due to packaging sterility (Bryant, 1988), debates regarding what size of defect allows microbes to penetrate the package and result in a breach of sterility remain. With the risks of failure being potential loss of human life and staggering negative economic impacts, these issues will likely remain very visible.

Consequently, those working in the medical industry face a daily balancing act between patient safety and cost competitiveness. Patient safety comes under increasing scrutiny as the frequency of nosocomial (hospital acquired) infections rise. The Centers for Disease Control and Prevention (CDC) estimate that approximately 90,000 to 100,000 patients die annually as the result of nosocomial infections. This represents the 4th leading cause of death in the United States according to Littell (2004), and the cost to treat the nearly 2 million occurrences is estimated to be $11 billion per year (Schierholz and Beuth, 2001) (Littell, 2004). Such statistics have resulted in programs aimed at reducing infection rates and brought everything from the genetics of microbes to common hygiene practices under scrutiny (Centers for Disease Control and Prevention [CDC], 2002). Adding to the concern surrounding infections is the indication that microbes are becoming more resistant to both processes and drugs (Berens, 2002; Ehrman, 1998; Rubin et al., 1999) that are intended to kill them.

Dr. Michael Burday, PhD, Director of the Division of Clinical Microbiology at the University of Medicine and Dentistry of New Jersey (UMDNJ)-University Hospital, states that the 'rate of infection is likely underreported due to the difficulty in tracking patients and reduced hospital stays.' Additionally, the types of organisms involved and

the causes of these infections are numerous, further complicating the tracking of nosocomial infections (Littell, 2004).

While the number of sterile medical devices that have become contaminated prior to use is unknown, it is known that devices contaminated with bacteria have the potential to cause harm (Littell, 2004). The more invasive a device, or the higher its regulatory classification (Table 1), the greater the risk. This fact was borne out in a study published in 1978 that estimated that 45 percent of all nosocomial infections were due to implant devices (Stamm, 1978). These increased rates of infection are likely due to the fact that invasive devices impair the patient's defense mechanisms and, when contaminated, can result in resistant chronic infection or tissue necrosis (Schierhola and Beuth, 2001).

Package Integrity Testing

As a result of these realities, package integrity or the ability of the package to prevent microbial penetration, especially when medical devices are involved, is increasingly important. Device manufacturers must package devices so they withstand "the rigors of shipping and arrive at the point of use in a safe and functional condition" (Spitzley, 2002). In addition, they must employ testing protocols that provide evidence that package integrity will be maintained from the sterilization process until delivery to the patient.

Current tests tend to be destructive and, therefore, costly. Only a fraction of the packages manufactured can be analyzed when destructive techniques are employed. Innovative integrity tests are needed to allow for increased sample sizes without increased destruction of packages.

New tests are emerging in response to this need. Various techniques are employed in the non-destructive detection of package defects, including: trace gas sensing, pressure/vacuum decay, mass-flow, and acoustic micro imaging. The greatly improved sensitivities of these new techniques (one system produced by Alcatel is touted to detect hole sizes of 100 Å [Leventon, 2001]) concern some in the device industry (Leventon, 2001; Spitzley, 2002). "As device packagers consider the alternatives, how do they know how much sensitivity is enough and how much is overkill?" (Leventon, 2001)

The required testing sensitivity level, in terms of the size of defect, that results in microbial contamination has been examined many times. The published limits fluctuate widely and, as a result, medical device manufacturers' current test protocols also vary greatly in terms of acceptance criteria (Gilliland et al, 2004). Without a pressure differential, published values for the sensitivity level vary from "between 0.2 micron to 0.4 microns" (Howard et al, 1980) to suggesting that it is likely that the critical leak size exceeds 10 microns (Lampi, 1980). Others indicate that the critical value is "less than 10 microns" (Blakistone et al., 1996; Harper et al., 1995; Hurme et al., 1997; Keller et al., 1996 and Keller, 1998), "10 or 5 microns" (Chen et al., 1991), "considerably larger than 1 micron" (Lake et al, 1985), 1 micron "under certain conditions" (McEldowney et al., 1990; Placencia et al., 1986), 11 microns (Lampi, 1980), all the way up to 22 microns (Gilchrist et al., 1989).

It is important to understand the variations in experimental methods that could help explain the observed ranges of values reported in the literature. These include:

- duration of time that the package is exposed to the microbes
- package geometry
- porosity of package components
- thickness of package components
- rigidity of the package at test
- method of microbial challenge (immersion, aerosol, talc and dynamic, or static)
- pressure differential across the sterile barrier
- types, sizes, states, and concentrations of the challenge organisms
- types of package defects and the methods of generating, and measuring, the defects.

Basis for Developing a New Method

The research conducted and presented in this dissertation provides answers to one specific aspect of the question posed above by Leventon (2001), regarding the level of sensitivity required for package integrity tests. That specific aspect is the determination of 'what effect does pressure differential, on the inside and outside of the package, have on microbial penetration of a rigid, non-porous tray intended to contain a sterile medical device?' The specific pressure differential examined simulates the descent of an aircraft from 8,000 feet, or a ground shipment descending from 8,000 feet. Two experiments were conducted to answer this question. Each of the experiments was conducted using a tray typical for the device industry.

It is understood that pressure differential is only one of a variety of factors that have the potential to influence microbial penetration, but it is believed that keeping the design

of the experiment as simple as possible is the best approach, and provides useful information to build future research.

The value of this research is great. One of the major reasons for packaging-related recalls in the device industry is listed by the FDA as, "Defective packaging: Potential for breach in sterility" (Food and Drug Administration, Center for Devices and Radiologic Health, QSR Manual, Section 820.70).

"Potential" becomes the operative term. If a manufacturer finds a defect, they remove the packaged product from distribution. It is removed, not because it has a known breach of integrity, but because there is a potential for it. As a safe, but costly, approach, when a defect is detected, the entire lot is likely to be pulled.

These types of recalls have many root causes including, but not limited to, machinery issues, material concerns, and the rigors of distribution. Little is known about precisely what size defect will allow for a breach of sterility, and what size poses no threat to the patient. As discussed earlier, there is a great deal of variation in results from previous research that contribute to confusion in the industry.

The ultimate outcomes of the body of work, which includes this research and research to follow, are to enhance patient safety and to reduce packaging costs. Answers regarding "what effect does pressure differential have on microbial penetration of a rigid, non-porous tray intended to contain a sterile medical device" help to ensure that:

- patient safety is maximized
- informed decisions can be made in the event of a potential recall situation
- a sensitivity benchmark is established for integrity testers
- costs are minimized (packages and products are not thrown out unnecessarily)

The maintenance of a sterile barrier, or package integrity, for packages that contain medical devices is paramount because package integrity is of critical importance to patient well-being. Package integrity is defined in ASTM F1327-98 as 'the physical capability of a given package to protect its contents with the desired level of protection over a defined period of service; for example, as a barrier to physical, microbiological, or chemical challenges'. ISO 11607-1 defines package integrity as the 'unimpaired physical condition of a final package.'

Package integrity in the device industry is generally tested in one of two ways: physical tests and microbial challenge tests, also called biotests.

Physical tests typically look for defects in the package, which may permit the passage of microorganisms. Physical tests include: visual inspection, destructive and non-destructive (ASTM F1886), dye penetration, destructive (ASTM F1929), bubble immersion, destructive (ASTM D3078, ASTM D4991, ASTM F2096), trace gas sensing techniques, non-destructive (ASTM F2391) for helium and (ASTM F2228) for carbon

dioxide, pressure decay, non-destructive (ASTM F2095) and vacuum decay, non-destructive (ASTM F2338), and acoustic microimaging, non-destructive.

Microbial challenges are based on the principle that 'a leaky container will allow bacteria to contaminate the container and its contents' (Marcy, 1992). Although there is a standard for challenging packaging **materials** with biological organisms, (ASTM F1608-00 'Standard Test Method for Microbial Ranking of Porous Packaging Materials [Exposure Chamber Method])', no ASTM standard currently exists for challenging an **entire package** with microbes.

As previously discussed, medical device packages must "withstand the rigors of shipping and arrive at the point of use in a safe and functional condition" (Spitzley, 2002). Professionals in the device industry must demonstrate, with a very high degree of confidence using objective evidence, that the integrity of medical device packages will be maintained throughout storage, handling, and distribution. This process is referred to as package validation (Global Harmonization Task Force, Process Validation Guidance, 1999).

In the past, validation typically utilized a whole package microbial challenge test that subjected the device package to standardized tests that simulated distribution and then challenged the packages with microbes via talc, aerosol, or immersion methods. After these steps, the outside of the package was disinfected thoroughly, and the package was opened and swabbed on the inside after which time the swab was cultured, incubated and examined for growth (Figure 2). However, this approach drew much criticism from the industry (Hansen, Jones et al., 1993; Spitzley, 1994; Scholla, Sinclair et al., 1995).

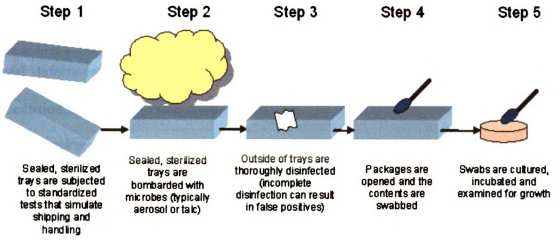


Figure 2 - Traditional Whole-package Microbial Challenge Test

This criticism was brought to light in a study conducted by the Health Industry Manufacturers Association's (HIMA) Sterile Package Working Group (Hansen, Jones et al., 1995). The study compared whole package microbial challenge testing to two physical tests in their ability to detect channels in the seals of packages. The two tests that HIMA compared with the whole package microbial challenge test were dye penetration and visual inspection.

When conducting the dye penetration test per ASTM F1929-98 (2004), a solution consisting of a wetting agent and an indicating dye is injected into the product-filled package. The orientation of the package is adjusted so that the dye is allowed to contact the seal edge for a minimum of 5 seconds and a maximum of 20 seconds. This is a simple, easy way to test for the presence of channels, a type of defect found in seals. Visual inspection is also a standardized test method (ASTM F1886), which can employ light and magnification in order to examine packages for defects.

The HIMA group rejected the null hypothesis, finding a significant difference existed between the physical methods and the whole package microbial test's ability to detect seal defects in sterile medical device packages. The physical test methods correctly identified all samples containing defects, and did not report any false positives. The whole package microbial challenge test "resulted in correctly positive findings [sic] of the test organism in only two instances. Equally important, the microbial challenge test also resulted in a false positive in one control sample and positive findings of organisms other than *B. subtilis* [the test organism] in four other instances" (Hansen, Jones et al., 1995).

The HIMA group criticized whole package microbial testing for a number of reasons. They indicated that the methods used in whole package microbial challenge testing were not reproducible or reliable, and that sophisticated personnel and facilities were needed to conduct testing of this type. They also indicated concerns about inadequate disinfection of the package after it had been exposed to microbes (Figure 2, Step 3). The group stated,

> "...the exterior of the package should be disinfected to reduce the probability of obtaining a false positive during sterility testing. What is an appropriate level of disinfection? Should a physical or chemical disinfection process be used? If a physical method, such as ultraviolet light is chosen, are the packaging materials UV transparent? The use of UV light may actually reduce the microbial population inside the package if the film component allows UV transmission. If a chemical disinfectant is chosen, does the liquid permeate the porous packaging material and does the material have sufficient

wet strength to withstand the chemical disinfection?" (Hansen, Jones et al., 1995)

The group also took issue with Steps 4 and 5 of the process (Figure 2, Steps 4 and 5):

> "...microorganisms that have entered the package must be recovered and cultured. If the surface area of the device is relatively small compared to the surface area inside the package, should additional techniques be used to improve recovery of microbes that have entered the package?" (Hansen, Jones et al., 1995)

Experiment One, New Method Development - Can we aseptically fill packages with a microbial growth medium without introducing microbes?

Experiment One is an alternative to the traditional method evaluated by the HIMA group, to more effectively measure the microbial penetration into packages. In this study, sealed, sterile device packages were aseptically filled with a known volume of sterile agar, exposed to an aerosol of microbes and then incubated to see if any microbial growth takes place. This reduced the probability of false positives, as packages are not opened to detect the entry of microbial cells. This technique also eliminated the need to disinfect the outside of the packages, another of the criticisms of the HIMA group.

In order to employ this technique to examine the impact of pressure differential on microbial penetration, described later in this dissertation, the new method needed to be verified. It had to be determined that the filling process did not significantly influence microbe penetration; i.e. that there was no significant difference between the sterility level of the agar and the sterility level of the agar-filled packages. (Reference Appendix I, New Method Development).

Primary Research- Applying the New Methodology

Experiment Two, Primary Research - What effect does pressure differential have on microbial penetration of a rigid, non-porous tray intended to contain a sterile medical device?

During the normal course of handling, packages experience very small pressure differentials across the sterile barrier that have the potential to act as a "driving force" for microbial penetration. It has been suggested that for microbial penetration to occur, a driving force must be present across the sterile barrier (Hackett, 2001). These driving forces are pressure differences that result from changes in the environment surrounding the package and are typically VERY small; for example, a calculation using Bernoulli's law[1] suggests that a package being moved at a rate of 3 feet per second through air experiences a pressure differential of 0.0000728 psi.

Microbial penetration is likely to occur even in a static environment, due in large part to pure diffusion (Brownian motion). Additionally, events in distribution, like descending from a mountain range or the ascent and descent of an airplane induce larger pressure differentials. In an article presented at HealthPack in March of 2001, Earl Hackett, a former Research Associate at Dupont, indicated the importance of the issue, "the flow of gas (and the particles it contains) into a package may be the most serious threat to the sterility of a packaged device" (Hackett, 2001).

How does the magnitude of the pressure differential influence microbial penetration into a package? It is tempting to say the larger the differential, the greater the microbial

---

[1] $\rho = \frac{\gamma v^2}{2g}$ where ρ = pressure, γ = density of air = 0.075 lb/ft^3 at standard temperature and pressure g = acceleration due to gravity = 32.2 ft/sec^2 v= wind speed in ft/sec

ingress. However, this neglects the possible effects of flow filtration theory, which have been shown to apply to porous membranes (Scholla et al., 2000). This theory states that a "moderate" flow rate maximizes microbial penetration. On the low side, the low flow rates that accompany diffusion is theorized to be the predominant mechanism of penetration. Under high flow rates, impaction predominates, and the microbes are removed because they are "smashed" into porous fibers. According to this theory, maximum penetration will occur when neither impaction nor diffusion is the predominant mechanism.

Theoretically, the flow of a gas through a cylindrical defect in a package under a negative pressure differential can be calculated using the equation $Q=A\Delta P\sqrt{(RT/2\pi M)}$ where: R=the molar gas constant, T=absolute Temperature, M=gas molecular weight, A=area of the orifice, and ΔP=pressure differential. This flow equation has been shown to be valid for pressure differentials down to 1 psi (Hackett et al., 2000). Research performed by Earl Hackett (Hackett et al., 2000) explored the effect of pressure differentials on microbial penetration into porous structures and demonstrated that greater permeability of the packaging material imparts a greater tolerance of package defects because the more permeable barrier material will allow more of the exchanged air to move through the material instead of forcing it through the defect. As a result of this finding, this research examines flow through a cylindrical orifice in a package composed entirely of non-porous material, a more severe and therefore, more conservative test of microbial penetration in light of Hackett et al's (2000) findings.

This research seeks to determine the need for a driving force other than that introduced by gravity or molecular motion for microbial penetration into a non-porous

package. Factors that have been held constant include: the orientation of the defect relative to the falling particles, the temperature and humidity of the test environment, the aerosol delivering the microbes, the test organism concentration (0 CFU/mL and 10^6 CFU/mL), and the exposure time of the packages to the aerosol. Thermal laser drilled holes in the test trays of 10 micron and 100 micron were compared to trays that did not have holes.

"A major cause of pressure differential during transport is the ascent and descent of transport aircraft" (Hackett, 2001). The descent phase results in a negative pressure differential on the packaging system, with the outside air exerting pressure on the surface of the package, and theoretically facilitating the penetration of outside contaminants into any defects in the packaging system. This research focused on the potential ingress of microorganisms as a result of a negative pressure differential. Test groups of sterile trays were exposed to an induced pressure differential simulating a package exposure when descending in an aircraft or ground shipment from 8,000 feet. Cargo and commercial aircraft have a pressurized cargo hold, usually maintaining pressures of 8,000 feet. An identical set of test groups were not exposed to an induced pressure differential, therefore, gravity served as the driving force.

CHAPTER 2

LITERATURE REVIEW

Historical Perspective on Testing

Whole package microbial challenge testing was first examined by Szczeblowski and Rubinate in 1965. They submerged pouches carrying 560 micron holes generated with hypodermic needles in a bacterial bath. Only half of the test pouches exhibited detectable microbial penetration. They concluded that some other action was needed in addition to submersion (Szczeblowski and Rubinate, 1968).

Based, in part, on Szczeblowski and Rubinate's results, Maunder developed a biotester that subjected pouches to a flex action while submerged (Maunder et al, 1968).

D. T. Maunder, J. F. Folinazzo, and J. J. Killoran - Continental Can Company and U.S. Army Natick Laboratories, 1968

Maunder's et al. (1968) simulated the handling abuse that a flexible food pouch might undergo when carried by a soldier. The handling was simulated by applying mechanical pressure to two areas of the pouch, which induced flexing. The researchers believed that the flexing would not only simulate handling but would 'remove minute particles of product that might be blocking a leak.' The test pouches contained radiation-sterilized chicken-ala-king. This product was selected because of its semi-fluid consistency, which the researchers believed would contribute more to pouch leakage than a solid food. The product also provided a good growth medium for the test organism, *Aerobacter aerogenes,* a gas-producing biotest organism. Organism concentration which the pouches were submerged in was reported to be in 'excess of 20×10^6 cells/ml.'

Defects were created in four ounce retort pouches using a sewing needle. The holes were placed a half inch from the top seal midway between side seals. Defect sizes ranged from 33 to 160 microns; the method of verification was not included.

Custom equipment was constructed which would allow application of mechanical pressure to the pouches, resulting in flexing while the pouches were immersed in water containing the test organisms at the aforementioned concentration. Biotesting lasted 15 minutes; this allowed for approximately 30 complete flexing cycles. The pouches were then removed and incubated for 3 weeks at 30°C. Approximately 10 percent of the pouches with holes did not show contamination. A microscopic examination indicated that, frequently, food product blocked the defects.

This team concluded that a biotest should be included in any package integrity program.

M. J. M Michels and B .L. Schram - Unilever Research Duiven, 1978

The main objective of Michel's and Schram's (1978) research was to determine the effect of handling procedures on contamination rates of pouches and not to determine the minimum hole size which allows microbial penetration. However, they did draw conclusions regarding hole size in relation to contamination rates. Their work was performed with 130 x 170 mm multi-layered pouches composed of a 12 micron polyethylene terephthalate (outer layer), 9 micron aluminum (middle layer), and 70 micron polyethylene (inner layer). The pouches were filled with 150 ml of yeast extract-peptone broth and sealed. The pouches were then heat processed with steam with an F_o value between 10 and 12.

Package integrity was evaluated by the use of immersion testing in a medium contaminated with *Enterobacter aerogenes.* The culture consisted of a concentration of greater than 10^8 cells/ml, and testing was performed at 30°C with an exposure time of 30 minutes. After pouches were exposed, they were incubated for a week or more, examined for swelling, and pH was determined to identify any pouches that experienced contamination. Each test group contained 10 pouches that were purposely punctured with a 100 micron needle. These pouches were intended to be positive controls to ensure the efficacy of the biological test. There was no characterization of the resultant hole made by the needle.

The sampling procedure included three post processing handling steps, each with 2 replicates, a total of 9 tests, with 1,180 deliberately punctured pouches. One hundred of these punctured pouches per handling procedure (300 pouches) served as positive controls and were checked for leakage with the biotest.

The positive control group (300 pouches) exhibited 100 percent contamination. Pouches from the worst case group (handling while wet) exhibited a 90 percent contamination rate. The contamination results dropped significantly as the handling procedures improved; drying after manually handling wet resulted in 10 percent contamination rates, and the group which was dried first and then handled dry only had a 1 percent contamination rate.

In this research, while the biotest was used only to test controls in the study, it illustrates that under worst case conditions, a 'large' induced defect in the pouch submerged in a high concentration of motile microbes may result in 100% contamination.

P. J. Anema and B. L. Schram - Unilever Research Duiven, 1979

Anema and Schram (1979) repeated the biotest preformed by Michels and Schram (1978), but instead of using a retort package, Anema and Schram used semi-rigid packages.

The packages were made by deep drawing a laminate material of approximately 100 micron aluminum with a polyolefin layer on the inside and a lacquer layer on the outside. The containers were closed horizontally, and the seal width varied between 1.5 and 4 mm.

The results revealed a very small amount of defective seals (0.3 percent to 0.8 percent). While the extent of the defect that resulted in the contamination of the tray was not reported, the study does indicate the viability of the biotest method for detecting seal defects in semi-rigid packages.

Robert R. Reich - Ethox Corporation, 1985

Reich's (1985) evaluated the microbial barrier properties of intact medical device packages exposed to a microbial aerosol test.

Ten devices from three lots of product were selected for the study. Each sealed package had been ethylene oxide (EtO) sterilized at least 30 months prior to the study. The devices were packaged in 0.020" clear polyvinyl chloride (PVC) thermoformed trays heat sealed with a coated 1073B Tyvek® lid.

A tesla coil was used to place a known number and size of holes in the Tyvek® lid. The voltage associated with the coil was used to control the size of the hole created. The holes were examined via scanning electron microscopy (SEM) for

homogeneity of size and shape. The positive controls in the study contained one hole of approximately 40 to 50 micron per 20 cm^2 of surface area.

The test organism used was *Bacillus subtilis* ATCC 9372. The spores were grown, harvested, cleaned, and incubated at 35+/- 2°C. The 'theoretical microbe chamber concentration was 40,000 per cubic meter.'

A Plexiglass® chamber was used for the test. The aerosol was formed using a nebulizer that produced particles of 0.3 to 2.0 mm, with some above 2 mm. A circulating fan was used to ensure distribution of the aerosol.

The exposure duration was 30 minutes. Following aerosol exposure, the product was exposed to 2 minutes of UV. The product was then aseptically transferred to 120 ml of Tryptic Soy Broth and incubated for 7 days at 35 +/- 2°C. During this period, the packages were observed for evidence of growth of the indicator organism.

The positive control trays (with created holes) demonstrated growth of the indicator organism. Under the conditions of the test, microbial penetration occurred in holes of approximately 40 to 50 microns. The results of the study indicated that the aerosol test method was a viable one and that the sterile medical device packaging was adequate to maintain a sterile barrier for the contained product.

Ana M. Placencia, Gordon S. Oxborrow, and James T. Peeler-1986

Placencia et al. (1986) illustrated the efficacy of an aerosol chamber method to test packaging materials. The Plexiglass® chamber used measured 19.1 cm wide x 21.0 cm high x 31.8 cm long. The challenge organism used was *Bacillus subtilis* var. *niger* in suspension at a concentration of 10^4 cells/ml. The organism was dispersed through the chamber with a nebulizer and a fan. The microbial exposure period was

15 minutes. Particle count was determined by drawing the aerosol through filter paper for a given period of time and counting the number of spores.

To determine the sensitivity of the system, 50 mm polycarbonate membrane filters with pore sizes of 0.8, 1, 2, 5 and 12 microns were used. There were 3 tests per pore size using concentrations of the test organism from 10^1 to 10^5 cells/ml. These data were also used to determine the probability of detection. The sensitivity evaluation study also included the challenge of heat seal coated 1073B Tyvek® of an area of 15.21 cm^2 containing 3 categories of microholes (0, 1, and 5 per area). Microhole diameter averaged 67 microns. Nine samples per category of hole quantity and microbial concentration (10^1 to 10^5 cells/ml) were evaluated.

The testing to evaluate the biobarrier properties of packaging materials included five different types of materials, each with an area of 15.21 cm^2 (Table 2).

Table 2 - Materials Examined by Placencia et al (1986)
45 lb autoclavable paper
44 lb machine finished surgical grade paper
42 lb blue surgical kraft paper
Uncoated 1073B Tyvek ®
Coated 1073B Tyvek ®

Each material contained three categories of microholes (0, 1, and 5 per area). The average microhole varied in diameter from 47 to 67 microns. There were 25 samples per category of microholes. The materials were exposed to '8.55 spores per square centimeter at an airflow rate of 2,831.7 ml/min for 15 minutes.' The airflow rate is

based on a pressure differential of 8.86 inHg (4.35 psi). The flow rate was based on the assumption that packaging materials are exposed to altitudes up to 3000 meters during ground transportation. A subset of the test materials and hole categories were evaluated at different flow rates; 1283 ml/min and 2831 ml/min.

The pore detectability study indicated that the method's detectability increases with the increase in microbial challenge concentration or the increase in pore size. The method detected a 1 micron pore at a concentration of 10^3 cells/ml in the polycarbonate membrane filters. The challenge detectability study with coated Tyvek® supported these results; the percent detection increased as the microbial concentration increased. The method detected differences among the materials. A significant difference was observed among materials and number of holes and between materials and number of holes. Uncoated Tyvek® was the most effective biobarrier. The flow rate evaluation indicated that as flow rates increase so does microbial penetration.

Robert R. Reich - Ethox Corporation, 1986

Reich (1986) repeated his 1985 chamber method to assess the biobarrier properties of packaging materials. This was analogous to the work conducted by Placencia et al, 1986. Reich (1986) employed a challenge condition of 5×10^4 microbial concentration of *Bacillus subtilis* spores at a pressure differential of 2 inHg for an exposure time of 30 minutes. This was considered an extreme test of the materials in question.

Reich concluded, as he did for the whole package challenge, in concurrence with Placencia et al.'s (1986) findings, that the aerosol chamber method is a viable method to evaluate the biobarrier properties of packaging materials.

P. Bankes and M.F. Stringer-The Campden Food Preservation Research Association, Chipping Campden, UK, 1988

Bankes et al. (1088) developed a model system to investigate physical factors that affect container leakage. Three types of organism were used as challenge organisms. They were *Pseudomonas* sp, *Bacillus* sp and *Staphyloccus aureus*. All were prepared into a final cell concentration of 1 x 10^9 cells/ml.

An apparatus was constructed with glassware to simulate a container. A membrane filter with pores of 1, 5 and 12 microns for simulated transient leakage channels and 5 microns for simulated permanent leakage channels was used in the model. Twelve micron diameter 'S' shaped channels were created with glass fiber bundles. A partial vacuum was applied to one of two pipettes containing liquid. Clamping was used on the mechanism to simulate permanent vs transient leaks. The vacuum range was 0 to 250 mmHg. This was accomplished by withdrawing air from the system with a syringe.

Researchers concluded that a 'positive correlation existed between microbial penetration and pressure differential'; the number of cells transferring through the 5 micron diameter permanent channels was much less than when a vacuum was applied.

James E. Gilchrist, Dhirendra B. Shah, Dave C. Radle, and Roger W. Dickerson, Jr.-Divisions of Microbiology and Food Chemistry and Technology, Food and Drug Administration, 1989

Gilchrist et al. (1989) compared trace gas testing, dye penetration testing, and biotesting when flexible pouches were subjected to each of the three tests. Biotesting was the accepted means of integrity testing at the time of this research; the intention of the team was to identify a faster, more practical alternative method to biotest flexible retort pouches containing food products.

The work was conducted with retort pouches that were 0.005" thick with dimensions of 6.5" x 8.5" and 12" x 14." Pouches were made of a trilaminate material consisting of polyethylene, aluminum, and polypropylene; a completely nonporous packaging system. The pouches were filled with Tryptic Soy Broth, sealed and then sterilized in a still retort.

The biotesting was performed by submerging the pouches in a Tryptic Soy Broth containing a 10^8 concentration of *E. coli 0A811.* The pouches remained in the broth for 2 hours and were agitated every 15 minutes. Following the submersion period, the pouches were rinsed with tap water and incubated at 37°C for 20 to 24 hours. Pouches that were contaminated were considered leakers. 'Leakers exhibited swelling, and contained a large concentration of carbon dioxide and hydrogen in the headspace, as well as turbid broth.'

Holes were introduced to the pouches by two methods. The first method was laser beam melting, producing holes from 17 to 81 microns in diameter. These holes were described as being approximately round with no tears or flaps. The second method was puncturing the pouch with a 0.004" diameter stainless steel wire. The

resultant range of holes produced was 22 to 175 microns. These holes were described as 'aperatures with tears and flaps.' In addition to microscopy, the hole sizes were determined by the rate of helium flow through the hole. The rate of helium flow was proportional to the square of the hole diameter at that pressure. Upon completion of the biotesting procedure, the holes were remeasured with microscopy.

The results of the study indicated that the helium test method was more consistent at identifying small leaks than the biotest or the dye test. The results of the biotest indicated that the test bacteria passed through holes down to about 22 microns.

S. McEldowney and M. Fletcher-Department of Biology, University of Warwick, Coventry and Department of Microbiology, Campden Food Preservation Research Association, Chipping Campden, 1989

McEldowney (1989) investigated the effect of physical and microbiological factors on container leakage through the development of a model system for container leakage. The extent of interaction between numerous factors was determined.

Various organisms were used in the study including *Acinetobacter calcoaceticus*, *Staphylococcus* sp., *Pseudomonas* sp., *Bacillus* sp, and a *Coryneform bacillus*. These organisms were isolated from processed food cans and conveyor belts in a food processing lab. *Staphyloccus aureas* was 'isolated from a nose swab'. Two bacteria, *Enterobacter aerogenes* and *Enterobacter cloacae*, were included in the study as representative organisms of those used in biotests.

Pure bacterial suspensions were of concentrations of 3-4 x 10^8, 6-8 x 10^8, 1-5 x 10^9, or 2-5 x 10^{10} cells/ml. Mixed suspensions were of a total cell concentration of 1-3 x 10^9 cells/ml. Monolayer films of organisms and biofilms were also prepared for test purposes.

At a flow rate of 500 microliter/min bacteria was leaked into a test chamber filled with a phosphate buffer solution at varying pressures (0 mmHg for 60 minutes, 51, 102, 152, 203, 254 or 305 mmHg) for 60 seconds.

The following factors were then evaluated:

- *Bacterial Morphology*-leakage of the test organisms was evaluated through a 120 micron straight channel.
- *Leakage Channel Size*-leakage of the test organisms was evaluated through straight leakage pathways with diameters of 1, 3, 5, 8, 10 and 12 micron.
- *Leakage Channel Shape*-leakage of the test organisms was evaluated through 15 micron diameter straight channels and 15 micron diameter convoluted channels.
- *Content viscosity*-the chamber was filled with phosphate buffer solution (control) or 4 percent pectin solution. Leakage of test organism was evaluated with 5 and 10 micron diameter straight leakage channels.
- *Bacterial Cell Concentration*-the pure cell test concentrations were evaluated through 15 micron diameter straight pathways.
- *Mixed bacterial populations*-the mixed cell suspensions were evaluated through 5 and 10 micron diameter straight leakage channels.
- *Monolayers and biofilms*-these were evaluated through straight leakage channels of 10 micron diameter.
- *Vacuum and exposure duration*- at a flow rate of 500 microliter/min bacteria were leaked into a test chamber filled with a phosphate buffer solution at 0 mmHg for 60 minutes, 51, 102, 152, 203, 254 or 305 mmHg for 60 seconds.

McEldowney (1989) observed that bacterial motility did not have a 'consistent effect on the rate of bacterial leakage.' For example, motile strains such as *Pseudomonas* sp did not penetrate at a faster rate than non-motile strains such as *Acinetobacter calcoaceticus* without vacuum. The effect of progressive increases in vacuum from 51 mmHg to 305 mmHg varied with bacterial species and morphology, but was greater in the presence of partial vacuum. Fluid flow through leakage channels also increased in the presence of vacuum. Leakage varied with vacuum, bacterial morphology, cell concentration, leakage channel size, and channel shape. The number of leaked cells was not proportional to vacuum or channel size. The effect of channel shape varied with bacterial species. Increased content viscosity decreased leakage. There was a difference in leak rates against vacuum between the pure bacterial suspensions and the mixed bacterial suspensions. The results indicated that there are three major factors influencing leakage; fluid availability, container vacuum, and content viscosity.

While these are the dominant factors, they are impacted by the other factors previously discussed. McEldowney's research clearly illustrates the complexity of understanding the container leakage process because of the wide range of interdependent variables.

C. Chen, B. Harte, C. Laf, J. Pestka, and D. Henyon-Michigan State University, School of Packaging and Department of Food Science and Human Nutrition, Pacteco U.S.A, and Pure-Pak, Inc, 1991

Chen et al (1991) evaluated the method of aerosol biotesting for determining the microbial integrity of aseptically filled juice packages as an alternative to microbial immersion testing.

A challenge test was run first using 30 ml sterile glass vials containing growth medium to determine the minimum hole size that the organisms could penetrate in a specific time period. Standard orifice sizes of 5, 10, and 15 microns were placed in the vial stoppers. Exposure times of 15, 30, 60, and 90 minutes were used for both the aerosol and immersion methods. The vials were then cleaned with chlorine solution and incubated. If turbidity in the growth medium developed after 48 hours, it was considered a positive result.

The test packages were 1.89 L flat-top cartons aseptically filled with apple juice. The cartons were nitrogen flushed resulting in a headspace composition of 97 percent nitrogen and 3 percent oxygen with dimensions measuring 19.7 cm x 13.3 cm x 7.6 cm (HxWxD). The juice boxes were composed of a lamination of polyethylene (PE/paper stock/Surlyn®/aluminum foil/PE). Standard pinhole orifices were used to create holes in the cartons. The orifices were 0.00254 cm thick and 0.635 cm in diameter.

The challenge organism was *Lactobacillus cellobiosus* (ATCC11739), an organism that can survive and reproduce at pH values below 4.5. A concentration of 2.5×10^6 cells/ml was used for all the tests.

Aerosolization occurred in a custom built Plexiglass® cabinet that contained 32 spray nozzles positioned throughout the cabinet. Packages were placed in the center of the chamber and the spray pattern was designed to cover the package geometry. Two exposure times were tested, 15 and 60 minutes. Following the microbe exposure period, the packages were sprayed with a 2500 ppm chlorine solution for 30 minutes. The packages were then removed and incubated at 35-37°C (ideal conditions for growth of the challenge organism) for 2 weeks.

The immersion test employed the same microbe and microbial concentration. The packages were submerged in the microbe suspension for 60 minutes. After the 60-minute period the packages were submerged in a 2500 ppm chlorine solution for 30 minutes, rinsed with tap water and incubated at 35-37°C for 2 weeks.

Detection of carbon dioxide, a product of the microbes in the headspace, indicated a likely breach in integrity. Additionally, containers were evaluated for a change in the pH level, and container swelling. Microbial growth in containers exhibiting these attributes was verified after the incubation period by removing the juice and performing standard colony counting.

"Five micron holes were detected after 30 minutes of spraying and 20 micron holes were detected after 15 minutes of spraying." The percentage of breached containers increased with increasing hole size. Sixty-two and one-half percent of containers tested positive for microbes when the 15 micron orifice was subjected to 60 minutes of spraying. Hole size was determined to be statistically significant, which indicated the aerosol method to be capable of differentiating between different orifice diameters. Test duration was not significant. Pinholes of 5 to 15 microns

were more easily detected with the aerosol method than the immersion method. The aerosol methods showed better detectability than the immersion test.

R. Ahvenainen, T. Mattila-Sandholm, L. Axelson, and G. Wirtanen - Technical Research Center of Finland, Swedish Packaging Research Institute, 1992

The objective of this research was to investigate 'the effect of microhole size and the food type on microbial penetration of aseptic plastic cups.' A driver to conduct the research was that, prior to 1992, very little research had been done on critical leak size for plastic containers. The majority of the research had focused on cans and retort pouches.

The test materials were high impact polystyrene cups with a volume of 115 cubic centimeters. Microholes were made in the bottom of the cup using a needle punch technique. These holes had diameters ranging from 5 to 100 microns, as measured by microscopy. Hole length was reported to be 50 +/- 10 microns. Seventy-five cm^3 of 3 different types of foodstuffs and soft agar filled the cups. The hole sizes tested with milk and agar ranged from 5 to 100 microns in diameter; for meat soup from 10 to 100 microns; and for sausage gravy from 25 to 100 microns.

The test cups were submerged in a container filled with *Enterobacter aerogenes* (ATCC 13048) suspension at a concentration of 10^7 CFU/mL. The chosen test organism is motile and is a gas producer. The submersion chamber, containing the cups, was then incubated and shaken for 30 minutes at 30°C. The cups were removed, wiped, and cleaned, and further incubated at 30°C for 1 to 6 weeks. Samples were taken at 1, 3 and 6 weeks and examined for microbial growth. Following incubation, the cups were carefully washed and examined microscopically

to see if the holes were blocked by the food contained in the cups. Bacterial growth was confirmed by the pour plate method.

Three hundred, sixty-eight cups were tested in total; 132 filled with soft agar, 72 filled with milk, 102 filled with meat soup, and 62 with sausage gravy. The smallest hole that the test bacteria could penetrate was 5 microns when the cup was filled with milk. The smallest hole for soft agar was 10 microns, for meat soup 20 microns, and sausage gravy 25 microns.

"The rate of penetration increased with lower viscosity product and increased with hole size." However, there were instances of 100 micron holes not being penetrated for all products.

The results illustrate that while bacteria did not penetrate certain samples, it did not guarantee package integrity. While a critical hole size for plastic containers cannot be determined from this study, it does indicate that the probability of microbial penetration is dependant on the product contained within and its viscosity.

Scott W. Keller, Joseph E. Marcy, Barbara A. Blakistone, George H. Lacy, Cameron R. Hackney, and Walter H. Carter, Jr. - Virginia Polytechnic Institute and State University, National Food Processors Association, and Medical College of Virginia, 1995

Keller et al (1995) examined the effects of test organism motility, test organism concentration, bioaerosol exposure time, hole diameter, and hole length with regard to their influence on microbial ingress into a flexible plastic pouch.

The retortable pouches used in the study were constructed of polyester (outer), Saran®, and cast copolymer polypropylene (inner). Nickel microtubes were used to create seal defects by sealing them in package heat seals. Four sizes of tubes were used; 10 and 20 +/- 2 micron internal diameter each with 5 and 10 +/- 1 mm lengths.

The pouches were filled with 99 ml of Tryptic Soy broth and retorted. They were allowed to thermally equilibrate to 25°C before testing.

Motile and mutated non-motile *Pseudomonas fragi* TM 849 were used as test organisms. Two source concentrations were evaluated. "The 10^2 cells/ml source yielded a final concentration of *P. fragi* in the Plexiglass® chamber of 1 CFU/cm^3 and 2 CFU/cm^3 for exposure times of 15 and 30 minutes, respectively." The 10^6 cells/ml source yielded a final concentration of *P. fragi* in the Plexiglass® chamber of 87 CFU/cm^3 and 178 CFU/cm^3 for exposure times of 15 and 30 minutes, respectively. The inoculated aerosol was generated in the chamber by nebulizers connected to two air cylinders, equipped with air-flow meters.

Prior to aerosol exposure, the defect area was exposed by cutting the top seam of the pouch. Four pouches, one of each defect size, were hung on a rack and placed in the chamber. Eight experimental sets were run for a total of 128 pouches. Each of the sets contained four combinations of the variables. Four replicates were run.

Six of the 128 pouches exhibited microbial contamination. All of the positive units were exposed to the motile, higher microbial source concentration of 10^6 cells/ml. These two variables were found to be statistically significant. The variables of channel length and exposure time were not statistically significant, but 'the interaction between an exposure time of 15 minutes and a 10 micron hole was statistically significant.'

Sixty-six percent of the positive results were in the lower probability group, the 10 micron hole size. It was concluded that the 'method of aerosolization can be used to detect microholes previously considered statistically improbable to detect'.

Researchers concluded that an aerosol challenge was more representative of conditions that the package would likely encounter in distribution, than that of microbial immersion testing, and that it was a viable means of assessing package integrity.

Carol L. Harper-National Center for Food Safety and Technology/FDA, 1995

Harper's (1995) efforts served to develop guidelines for a new, off-line, non-destructive package integrity test system. Specifically, the research team needed to determine the minimum channel diameter that would allow microbial penetration.

A motile organism, *Enterobacter aerogenes*, chosen because it exhibits robust growth, was used at a concentration of 1×10^9 CFU/mL. Seal defects were created by sealing tungsten wire, of known diameters, into the seals of a flexible plastic. The seal and the area around it was then cut out and placed into a filter holder. The edges were sealed with silicone to avoid any leakage around the test material. Once the material was sealed, the apparatus and the lower chamber holding 250 ml of medium was autoclaved. While the material was still warm, the wire was removed, leaving channels from 10 to 125 microns in the seal area. The diameters of the channels were verified by microscopy. The apparatus was then incubated overnight to ensure sterility. Solution inoculated with the challenge organism was poured into the top reservoir of the apparatus. If a sample exhibited contamination, the 'apparatus was bubble tested to verify that there were no leaks in the filter ring seal'.

A growth curve was generated to determine the time needed for one colony to multiply to a level that would be detectable by plating methods. This time was determined to be 4.5 hours. After preliminary experimentation, Harper (1995) set the

final duration for the experiment at 24 hours. The test organism was 'capable of penetrating channel leaks of a 10 micron diameter,' channel length was not reported.

Barbara A. Blakistone, Scott W. Keller, Joseph E. Marcy, George H. Lacy, Cameron R. Hackney, and Walter H. Carter, Jr-National Food Processors Association, Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Medical College of Virginia, Virginia Commonwealth University, 1995

The purpose of this research was to examine the critical defect threshold dimensions by using artificially created channel leaks of 10 and 20 microns across seals that were 5 and 10 mm in length in plastic pouches.

The pouch material was polyester-polyvinylidene chloride-cast copolymer polypropylene. Microtubes were positioned in the seals to create the channels. Another seal was placed above the microtubes to protect them from contamination until use. The pouches were filled with 100 ml of Trypticase soy broth and then retorted.

The test organism, *Pseudomonas fragi,* was prepared at concentrations of 10^2 and 10^6 CFU/mL. Both motile and non-motile variation of this organism were evaluated. The seal on the pouch was cut to expose the channels. The pouches were placed in a sterile container and immersed in the test organism suspension. The container was gently rocked every 5 minutes. The exposure duration was 15 and 30 minutes. Following immersion testing, the media contained in the pouches was cultured to determine if the test organism had penetrated.

The results indicated 56 of 128 pouches tested (44 percent) were positive. Analysis showed that channel length had no influence on the positive results. Microbe concentration level (10^2 and 10^6 CFU/mL) and the interaction with time

were found to be statistically significant ($P<0.05$). Exposure time as a sole factor was not statistically significant nor was channel diameter. "Approaching statistical significance was the relationship of concentration and motility versus time." The increase of exposure time had a slightly negative effect on contamination with motile organisms, while not statistically significant. Motility appeared to be more of a positive factor at the lower microbial concentrations than the higher concentrations.

Blakistone et al. (1995) concluded that biotesting can be "misleading if not designed for the particular exposure conditions of the package." While Blakistone et al. (1995) did not draw any conclusions regarding the critical defect threshold, they did emphasize the need for a "model for risk assessment of packages" under specific conditions.

Joseph E. Marcy-Food Science and Technology, Virginia Polytechnic Institute and State University, 1995

Marcy (1995) investigated the viability of an immersion biotest for flexible retort pouches.

The pouches used in this study were Meals, Ready to Eat (MRE) retortable pouches. The laminate consisted of 48-gauge polyethylene terephthalate (PET)/adhesive/0.00005 inch foil/adhesive/0.003 inch polypropylene (PP). The pouches contained 8 ounces of product and were 4 ¾ x 8 ½ inches in size.

Channel leakers were manufactured in the seals by placing a 20 micron Teflon® fiber in the seal area prior to sealing. After cooling, the fiber was removed from the pouch. Another set of leakers was produced using platinum wires 100, 130, and 210 microns in diameter. The size of the Teflon® fibers was verified with an optical microscope and the size of the platinum wires was verified with a micrometer. The

20 micron hole size was verified microscopically before and after the biotest resulting in a size of 20 ± 1 micron. The larger holes were tested by using an electrolytic test and correlating the diameter of a leaker to the intensity of the current flow.

Five sets of retortable pouches were manufactured for biotesting; 150 MRE pouches containing 20 micron channels and three sets of 50 MRE pouches with channels of 100, 130, and 210 microns. The fifth set did not contain any manufactured leaks. All pouches were double sealed to protect the channels from contamination until the biotest. Each pouch contained 80 ml of Tryptic Soy Broth.

The test organism was gas producing *E. coli* 9637 at a concentration of 4×10^8 cells/ml. The flanges of the test pouches were wiped with the test organism broth before immersion to ensure adequate wetting of the seal. The pouches remained immersed in the suspension for 2 hours and agitated every 15 minutes. Following the test, the pouches were rinsed with tap water and incubated at 37°C for 24 to 48 hours. A positive biotest was indicated by swelling of the pouch and turbidity of the broth. A positive result was confirmed by analyzing the gas content of the headspace. To ensure the only leak present was the manufactured leak, the units were dye tested.

The results indicated that 100 percent of the larger sized holes (100, 130, and 210 micron) exhibited contamination and 96 percent of the 20 micron hole pouches exhibited contamination.

The researchers concluded that 20 micron leaks result in bacterial penetration. They also concluded that channel leak length does not affect the likelihood of

contamination since there was no difference noted with 20 micron channels of 5 mm and 127 microns in length.

Matthew Joseph Gibney IV- Department of Food Science and Technology, Virginia Polytechnic Institute and State University, 2000

Employing test methods similar to Keller (1998) and Blakistone (1995), Gibney compared air-filled package defects with fluid-filled defects to determine the critical leak size for air-filled defects. Gibney used a bioaerosol chamber to expose the packages to the test organism. Nickel microtubes were employed to create channel defects of 2, 5, 7, 10, 20, and 50 microns in diameter. Defect sizes and pressure differentials were examined for significance. Pressure differentials of 0, -6.9, -13.8, and -34.5 kPa were employed. The test organism used was *Pseuduomonas fragi* at a concentration of 10^6 cells/ml.

Gibney (2000) referred to defects in packages that contained only air as 'air-filled microholes'. He compared penetration into these holes with penetration into microholes that were situated against liquid product. Gibney concluded that the critical leak size of an air-filled microhole was approximately 7 micron for pressure differentials ranging from 0 to –34.5 kPa. This differs from previous research with liquid filled defects in which the critical defect coincided with the threshold leak size or the 'defect size which allows the initiation of leakage at a particular pressure' (Keller, 1998).

ASTM F 1608 – 00 Standard Test Method for Microbial Ranking of Porous Packaging Materials (Exposure Chamber Method), 2000

This method is intended to compare the ability of porous packaging materials intended for use in medical device packaging to filter airborne bacteria. Samples of porous materials are subjected to an aerosol of *Bacillus subtilis* var *niger* spores at a challenge concentration of 1×10^6 cells/ml in an exposure chamber. Spores that pass through the porous material are collected and enumerated.

The materials are exposed to the aerosol for 15 minutes at a flow rate of 2.8 L/min. The materials are removed and the filter paper cultured and the plates enumerated.

The resultant data assessed the relative potential of materials to contribute to the loss of sterility of the product contained in the package. It is not intended to predict the performance of a material for a specific application.

Whole package microbial challenge testing was used extensively in the mid-1960s and 1970s during the development of the retort pouch (Lampi, 1981). As retort pouches began replacing cans for the containment of food products, the assessment of the bio-barrier changed to incorporate the stresses of package handling. Maunder tested pouches being flexed as they were exposed to immersion in a bath of microbes in order to simulate the handling of Meal Ready-to-Eat pouches by soldiers in the field (Maunder et al, 1968).

At the same time that the food industry was testing the integrity of retort pouches, medical devices were evolving. Prior to the 1970s, medical devices were sterilized in the hospital and re-used. This changed with the advent of the disposable device. Disposable medical devices were (and are) packaged and sterilized by the device

manufacturer, who, in turn, become responsible for the product's sterility from manufacture to arrival at the hospital. In parallel with these device advancements, a proliferation of new packaging developments also occurred. These developments involved the introduction of flexible materials for use in flexible pouches, and materials to package devices in thermoformed trays (Spitzley, 1994).

As the industry changed, the need for standardized packaging integrity testing became increasingly apparent. Publications in the early 1980s (Reich, 1985; Placencia et al, 1986) indicate that microbial challenge methods were the recommended method for testing the integrity of device packages.

Validation protocols for medical device packaging typically involved a whole package microbial challenge test. This was the case as recently as the 1990s. See Figure 2 for a complete description of the typical procedures of the past. As discussed, the whole package microbial challenge testing method drew much criticism from the industry (Hansen et al, 1995; Spitzley 1994; Scholla, Sinclair et al, 1995).

When presented with the results of the work of the HIMA group, the FDA agreed that physical test methods were 'superior to microbial challenge tests for sterile package integrity' (Spitzley, 2002) and began recognizing both types of tests for the evaluation of package integrity. The focus of the medical device packaging community shifted to the enhancement, standardization, and validation of physical test methods. Current tests (reference **Basis for Developing a New Method** Section of the Introduction, Chapter 1) tend to be destructive and, therefore, costly. Only a fraction of the packages manufactured can be analyzed when destructive techniques

are employed. Innovative integrity test methods were needed to allow for increased sample sizes without increased destruction of packages.

Emerging techniques such as: trace gas sensing, pressure-decay, mass-flow, and acoustic micro-imaging have greatly improved sensitivities. Acoustic micro-imaging has been shown to detect sub-micron gaps in test materials (Allen, 2002). The greatly improved sensitivities of these new techniques concern some in the device industry (Leventon, 2001; Spitzley, 2002). "As device packagers consider the alternatives [to traditional integrity tests], how do they know how much sensitivity is enough and how much is overkill?" (Leventon, 2001) This is an important question to the industry. A test that passes holes that allow microbial penetration has the potential to negatively impact patient health. However, tests that fail packages that do not present health risks needlessly drive up costs when packages and products are destroyed unnecessarily.

The intuitive response to the question of the appropriate sensitivity levels is that if the hole is larger than the microbe, there is a potential for penetration. However, this seemingly simple answer does not hold true. Various researchers have explored the question, "What is the smallest defect that allows microbial penetration into sterile packages?"; with widely varying results, as previously discussed (Maunder et al, 1968; Michels et al, 1978; Anema et al, 1979; Reich, 1985; Reich, 1986; Placencia et al, 1986; Bankes et al, 1988; Gilchrist et al, 1989; McEldowney et al, 1989; Chen et al, 1991; Ahvenainen et al, 1992; Keller et al, 1995; Harper, 1995; Blakistone et al, 1995; Marcy et al, 1995; Gibney, 2000).

A natural consequence of the numerous factors available for investigation is that the experimental objectives vary significantly, as do the experimental designs that researchers working in this area employ.

Varying Objectives

Maunder (1968), Marcy (1995), and Michels (1978) investigated the viability of immersion biotests as an indicator of package integrity for retort pouches, and to examine the impact of handling on microbial penetration (Maunder, 1968; Marcy, 1995; Michels, 1978). Anema also investigated the use of immersion biotesting, but used rigid trays (Anema et al, 1979). Gilchrist compared the effectiveness of immersion biotesting with helium and dye penetration testing on flexible pouches (Gilchrist et al, 1989). Ahvenainen used immersion biotesting to evaluate the relationship of different foods contained in aseptic cups and the microhole size on bacterial contamination (Ahvenainen et al, 1992). Blakistone's research was directed at determining the size of microhole, which resulted in microbial contamination of plastic pouches when immersion tested (Blakistone et al, 1995). Harper's intent was to develop guidelines with respect to the required sensitivity for non-destructive package integrity testing; this work employed packaging **materials**, where many of the others involved an evaluation of **the entire package** (Harper, 1995).

A team led by Chen evaluated the use of bioaerosol testing as an alternative to immersion testing for evaluating aseptic juice packs (Chen et al, 1991). Reich (1985, 1986) evaluated the use of aerosol testing for medical device trays and in a separate study evaluated the use of aerosol testing for packaging materials (Reich, 1985; Reich, 1986). Placencia also used biotesting and investigated packaging materials as well as whole

package testing (Placencia et al, 1986). Keller and Gibney evaluated numerous physical and microbiological variables with regard to their effect on microbial penetration and critical hole size using bioaerosol testing (Keller et al, 1995; Gibney, 2000).

And finally, Bankes and McEldowney studied various microbiological and physical variables effecting microbial contamination by the development of model systems and apparatus, which allowed them to study the variables and their interactions (Bankes et al, 1985; McEldowney et al, 1989).

Because of the varying objectives of different research teams and the resultant disparity in each of their experimental designs, it is not surprising that conclusions regarding threshold vary greatly as well.

Varying Results

Many researchers report the threshold for microbial penetration is less than 10 microns. Howard et al, (1980) indicated that the penetration threshold was 0.2 micron. Under certain circumstances, 0.2 micron rated filters allowed passage of certain bacteria (Howard et al, 1980). Blakistone et al, (1996), Harper et al, (1995), Hurme et al (1997), Keller et al, (1996) and Keller (1998) all indicate "less than 10 microns." Chen et al (1991) indicated it to be "10 and 5 microns." (Chen et al, 1991) Gibney (2000) reported 7 microns (Gibney, 2000), and Lake et al. (1985), reported "considerably larger" than 1 micron while both McEldowney (1990) and Placencia reported 1 micron "under certain conditions" (McEldowney et al, 1990; Placencia et al, 1986).

Other researchers support the idea that the penetration threshold is greater than 10 microns. Lampi (1980) indicated that it was **not likely** that the critical leak size was less than 10 microns. Gilchrist et al (1989), reported 22 microns, Reich (1985) indicated

a range of 40-50 microns and Axelson et al (1990), using an electrolytic test, determined the critical hole size to be 80 microns.

As mentioned previously, variation in experimental objectives and subsequent experimental design contribute significantly to the reported results. Tables 3 and 4 summarize physical variables (Table 3) and microbiological variables (Table 4) employed by the cited teams.

Table 3 - Physical Variables in Published Research

Researcher/ Year	Whole Pkg, Mt'ls or Model	Whole Package Description	Material Description	Product Contained	Defect Creation Method/ Verification
Maunder, 1968	Package	Flexible Pouch	Trilaminate	Chicken-ala-king	Needle/ Microscopic 33 to 160 micron
Michels, 1978	Package	Flexible Pouch	Trilaminate	Growth Medium	Needle/No Verification Cited 100 micron
Anema, 1979	Package	Rigid Tray/ Flexible Pouch	Trilaminate	Growth Medium	Needle/No Verification Cited 100 micron
Reich, 1985	Package	Rigid Tray	PVC/Tyvek	Device	Tesla coil/SEM 40-50 micron
Reich, 1986	Material	N/A	Various	N/A	Tesla coil/SEM 40-50 micron

Table 3 (cont'd)					
Researcher/ Year	Whole Pkg, Mt'ls or Model	Whole Package Description	Material Description	Product Contained	Defect Creation Method/ Verified
Placencia, 1986	Material	N/A	Various	N/A	Pores and Microholes from 47 to 67 micron diameters. Microholes present in various packaging materials/ Microscopic
Bankes, 1988	Model	N/A	N/A	N/A	1, 5, 12 micron pores to simulate transient leaks and 5 micron to simulate permanent leaks
Gilchrist, 1989	Package	Flexible Pouch	Trilaminate	Growth Medium	Laser Beam-17 to 81 micron in diameter Wire Puncture-22 to 175 micron in diameter-Verified by microscopy and flow rates
McEldowney 1989	Model	N/A	N/A	N/A	Numerous
Chen, 1991	Package	Aseptic Package	Trilaminate	Juice	Standard Pinhole Orifices 5 to 20 micron/No Verification Cited
Ahvenainen, 1992	Package	Aseptic Cups	High Impact Poly-styrene	Foodstuffs	Needle-5 to 100 micron/ Microscopy

Table 3 (cont'd)					
Researcher/ Year	Whole Pkg, Mt'ls or Model	Whole Package Description	Material Description	Product Contained	Defect Creation Method/ Verified
Keller, 1995	Package	Flexible Pouch	Plastic Trilaminate	Growth Medium	Microtubes-10 and 20 micron diameter and 5 and 10 mm length/No Verification Cited
Harper, 1995	Material	N/A	Flexible Plastic	N/A	Wires in seal-channels 10-125 micron/Micro-scopy
Blakistone, 1995	Package	Flexible Pouch	Plastic	Growth Medium	Microtubes-10 and 20 micron diameter and 5 and 10 mm length/No Verification Cited
Marcy, 1995	Package	Flexible Pouch	Trilaminate	Growth Medium	Fiber and Wires in Seals-20, 100, 130, and 210 micron/ Microscopy and Electrolytic Test
Gibney, 2000	Package	Flexible Pouch	Plastic Trilaminate	Growth Medium	Microtubes-2, 5, 7, 10, 20 and 50 micron/No Verification Cited
ASTM F 1608-00	Materials	N/A	Porous	N/A	Pores

Table 4 – Microbiological Variables in Published Research					
Researcher	Exposure Duration	Biotest Method	Driving Force	Challenge Organism	Organism Concentration
Maunder, 1968	15 minutes	Immersion	Flexing	*Aerobacter aerogenes*	20×10^6 cells/ml
Michels, 1978	30 minutes	Immersion	N/A	*Enterobacter Aerogenes*	10^8 cells/ml
Anema, 1979	30 minutes and varied	Immersion	N/A	*Enterobacter Aerogenes*	1.3×10^2 to 1.3×10^8 cells/ml
Reich, 1985	30 minutes	Aerosol	N/A	*Bacillus subtilis*	4×10^5 cells/m^3
Reich, 1986	30 minutes	Aerosol	N/A	*Bacillus subtilis*	5×10^4 cells/m^3
Placencia, 1986	15 minutes	Aerosol	Flow Rate	*Bacillus subtilis* var *niger*	10^1 to 10^5 cells/ml
Bankes, 1988	N/A	N/A	Vacuum	*Pseudomonas* sp, *Bacillus* sp, *Staphyloccus aureus*	1×10^9 cells/ml
Gilchrist, 1989	2 hours	Immersion	Flexing	*Escherichia coli 0A811*	1×10^8 cells/ml
McEldowney, 1989	Numerous	N/A	Vacuum	*Acinetobacter calcoaceticus Staphylococcus* sp., *Pseudomonas* sp., *Bacillus* sp., *Coryneform bacillus Staphyloccus aureas Ent. aerogenes Ent. Cloacae*	Multiple
Chen, 1991	15 and 60 minutes	Aerosol and Immersion	N/A	*Lactobacillus cellobiosus*	2.5×10^6 cells/ml
Ahvenainen, 1992	30 minutes	Immersion	Agita-tion	*Enterobacter Aerogenes*	1×10^7 cells/ml
Keller, 1995	15 and 30 minutes	Aerosol	N/A	*Pseudomonas fragi*	1×10^2 and 1×10^6 cells/ml

Table 4 (cont'd)					
Researcher	Exposure Duration	Biotest Method	Driving Force	Challenge Organism	Organism Concentration
Harper, 1995	24 hours	Immersion	N/A	*Enterobacter Aerogenes*	1×10^9 cells/ml
Blakistone, 1995	15 and 30 minutes	Immersion	N/A	*Pseudomonas fragi*	1×10^2 and 1×10^6 cells/ml
Marcy, 1995	2 hours	Immersion	Agita-tion	*Escherichia coli 9637*	4×10^8 cells/ml
Gibney, 2000	15 and 30 minutes	Aerosol	Vacuum	*Pseudomonas fragi*	1×10^6 cells/ml
ASTM F 1608-00	15 minutes	Aerosol	Flow Rate	*Bacillus subtilis* var *niger*	1×10^6 cells/ml

Variables

These research teams, and those reviewing their work, place varying levels of emphasis on the many factors that play a role in microbial ingress, largely based on the objectives of their respective studies. A discussion of the importance of the variables, based on conclusions drawn from their research follows.

Research has established that a driving force is required for microbial ingress (Hackett, 2001; Scholla et al, 2000; Floros, 1994; McEldowney et al, 1990; Ahvenainen et al, 1992; Placencia et al, 1986; Purohit, 1995; Harper, 1995; Maunder et al, 1968; Tallentire and Sinclair, 2002). Results from a model system developed by Bankes and Stringer supports the idea that the amount of leakage with a 5 micron hole varied with the amount of vacuum present. Their results support findings by Put et al (1972) that a can with a vacuum of 400 mmHg is more susceptible to leakage than one at atmospheric pressure (Put et al, 1972). In other words, a larger differential results in greater penetration through the package. McEldowney (1990) also developed a model system to study container leakage. She indicated that the effect of progressive increases in vacuum

from 51 mmHg to 305 mmHg varied with bacterial species and morphology (McEldowney et al, 1990). Gibney's (2000) research indicated that pressure did not play a significant role in microbial ingress, but that hydraulic diameter effects were significant (Gibney, 2000).

Keller et al (1995), reported factors critical to maintenance of the sterile barrier to be: defect dimension, organism concentration, and exposure time (Keller et al, 1995).

Floros (1994) discussed the comparison of critical hole size for different types of packaging systems. He indicated that a comparison cannot be made between cans and aseptic packages because the headspace for aseptic packaging, which is at atmospheric pressure, versus that of cans, is below atmospheric pressure (vacuum) impacts the results. The driving force for bacterial penetration is greater for cans than for aseptically packaged products. He hypothesized that this difference in driving force, would result in a smaller penetration threshold for cans than aseptic packages (Floros, 1994).

Michels (1978) determined that wet handling of post-processed retort pouches resulted in a significant increase in microbial penetration compared with pouches dried before handling (Michels et al, 1978). This would seem to indicate that moisture may serve to enhance the movement of contaminants though the sterile barrier.

Anema and Schram (1979) determined that microbial concentration had a significant effect on the percentage of packages contaminated (Table 5) with an immersion biotest, for both flexible and rigid packages. Higher concentrations of microbes resulted in 100 percent of package contamination. The results below are for flexible pouches with 100 micron holes.

Table 5 - Percent of Flexible Pouches Found to have Contamination at Varying Challenge Concentrations (Anema and Schram—1979)	
Concentration of Microbes cells/ml	Percentage of Contamination
130	18
20,000	52
520,000	100
130,000,000	100
Cooling Water	4.2

Additionally, they evaluated the effect of exposure time on the rate of contamination (Table 6). The results indicate that this is an important factor for consideration when designing a microbial challenge test (Anema et al, 1979).

Table 6- Percent of Flexible Pouches Found to have Contamination at Varying Exposure Times (Anema and Schram—1979)		
Exposure Time (minutes)	Concentration of Microbes cells/ml	Percentage Contaminated
1	1.5×10^4	15
5	4.0×10^4	58
15	4.6×10^4	57
30	2.3×10^4	45

Blakistone (1995) and Harper (1995) determined that the threshold defect size is dependent on the type of microbial challenge test that is used to test packages. This team concluded that immersion biotesting, which actually submerges a package in a bath of microbes, represents extreme conditions, and is not representative of the production and distribution environments for all package types. It was, however, indicated to be a good representation of the processing faced by retort pouches, which do experience an immersion procedure.

Blakistone (1995) and Harper (1995) also reported defect type as an important factor. In addition to channel leaks, blisters, wrinkles, incomplete seals, abrasion and punctures factor into the probability assessment of microbial ingress.

Additionally, Blakistone (1995) and Harper (1995) noted organism type as an important factor impacting microbial ingress. Researchers cited attributes such as size and shape, and motility as important determinants. As with Anema and Schram (1979), Blakistone (1995) and Harper (1995) indicated the concentration of the organisms in the test environment to be a critical factor. This was regardless of whether the organism was a vegetative cell or a spore, and was also found to be true for bacteria, yeasts and molds (Blakistone et al, 1995; Harper, 1995).

Marcy (1995) examined the defects themselves as having the potential to impact the penetration threshold. He found that factors such as hole diameter and surface properties, growth potential in the vicinity of the hole, and bacteria concentration were significant effects. He concluded that channel leak length does not affect the likelihood of contamination in his research with biotest immersion and flexible pouches (Marcy, 1995).

Scholla (2000) hypothesized that the speed of the particles containing microbes to be a significant factor. Scholla indicated that microbes exist as discrete particles, bound together and bound to other particles, typically present as an aerosol, and that complexity exists with the function of the aerodynamic particle size, which has to do with the mass and dimensions of a particle and its face velocity.

He employed flow filtration theory to explain the behavior of these complex systems. Filtration theory indicates that particles that are moving as a result of nothing more than

Brownian motion will penetrate materials via diffusion. Particles traveling at great rates of speed are likely to experience inertial impaction, be filtered when they collide with package materials at high rates of speed. In between these two extremes is interception, the point where neither diffusion nor inertial impaction dominates; it is this mechanism that is hypothesized to bring about the greatest microbial penetration. Added complexity exists when all three (interception, inertial impaction, and diffusion) filtration mechanisms happen at the same time (Scholla, 2000).

Factors affecting diffusion capture include a decrease in particle velocity resulting in higher retention times (pressure differential, flow rate). Electrostatic charge affects diffusion capture. This has significant ramifications to the device industry, as radiation, a type of sterilization technique for medical devices, is known to eliminate electrostatic charge on objects (Scholla, 2000). Bacteria tend to posses a negative charge (Brooks, 2004), thus the net negative charge of the bacteria and the packaging materials would likely result in the repulsion between the bacteria and the materials. The introduction of the aerosol in the cabinet would 'bleed' off the negative charge thus reducing the repelling forces.

Lake (1985) found the effect of post processing contamination a significant factor. This team stated that it is reasonable to assume that, theoretically, a 1 micron hole would be the smallest hole that would permit the passage of a bacterial cell if one considers only a particle size relationship to the opening. However, the situation is quite different when considering the parameters for post-processing spoilage in canned foods in which a liquid is necessary to transport the infecting organism into the can. The size relationship is an oversimplification of the situation because it does not account for physical properties

such as viscosity, temperature, surface tension, volatility, capillary pressure, etc., present in a liquid system. This team considered the following factors to be important: Whether the bacteria is in a chain, or clumps, bacterial encapsulation, the presence of flagella or other cell appendages, the presence of electrostatic forces and the physical characteristics of the leak itself. Based on these factors, Lake (1985) states that the smallest critical hole size for the post processing contamination of canned foods would be considerably larger than 1 micron (Lake et al, 1985).

The objective of McEldowney's (1989) research was to investigate the effect of physical and microbiological factors on container leakage through the development of a model system for container leakage. The extent of interaction between numerous factors was determined. Physical factors such as container vacuum, content viscosity, leakage channel size and shape were evaluated. Microbiological factors such as cell morphology and motility, cell concentration, pure and mixed bacterial suspensions, biofilms and monolayer films of organisms were also evaluated. The results indicated that there are three major factors influencing leakage; fluid availability, container vacuum, and content viscosity. While these were considered the dominant factors of this research, they were also impacted by the other factors previously discussed. McEldowney's research clearly illustrates the complexity of understanding the container leakage process (McEldowney et al, 1989).

Put's (1972) research focused on the mechanism of bacterial infection of canned foods using a bacterial immersion testing. This team also examined the influence of can construction and handling on microbial penetration. Several factors were determined to contribute to the re-infection of canned foods. The factors include the construction of the

can which incorporates several measurable parameters, the size, shape, length, profile of the leak, and the capillary action within the passage, the concentration of the microbial suspension, their motility, and reproductive rates, the time the can seams were exposed to the contaminated liquid, the viscosity of the contaminated liquid, the atmospheric pressure within the can after sterilization and cooling, handling, and sudden pressure fluctuations that may occur during sterilization and cooling.

Put's team concluded that bacterial re-infection of the cans resulted from passive transport, and occasionally active transport, of bacteria through a capillary passage. The efficiency of this transport is influenced by the viscosity of the suspension, the ability of the organisms to reproduce during the test and the ability of the passage to filter out organisms (Put et al, 1972).

Ahvenainen (1972) recognized that while previous research regarding critical hole size on cans and retort pouches cannot be applied to plastic containers, many variables involved apply to both types of packages. The variables he cited include vacuum, bacterial morphology, cell concentration, and leakage channel shape. The results of this research indicate that the probability of microbial penetration is dependent on the type and viscosity of the product contained within the plastic container (Ahvenainen et al, 1992).

Kamei et al (1991) examined microbial penetration for aseptically packaged milk in paperboard cartons. The focus of their research was to compare the difference in microbial penetration of cartons containing a high fat product, milk, and a low fat product, coffee. They placed pinholes (0.2 to 0.3mm diameter) in the board with sewing needles. They found that an increase in fat content in the contained product resulted in

higher rates of contamination through pinholes. They found no difference in samples with only one induced pinhole and those with 10 induced pinholes (Kamei, 1991).

Placencia's et al (1986) objective was to illustrate the efficacy of an aerosol chamber method to test packaging materials. They discussed the following factors as contributing to the microbial penetration of a package; temperature changes, transportation, and handling, storage conditions, altitude changes, changes in pressures during surgical procedures, and microholes in the material, sterilization processes, and any conditions that may result in 'bellowing' of the package (Placencia et al, 1986).

Bankes and Stringer (1988) developed a model system to evaluate the mechanisms of food container leakage. Factors they concluded to affect the leakage of microorganisms into the container included the presence of vacuum, the size and shape of the leakage channel, and volume of liquid passing through the channel and the test organism used (Bankes and Stringer, 1988).

Purohit et al (1995) stated, 'To our preconceived notions of size as a restriction to microbial ingress, we need to add the parameters of tortuous path, mean free path length, pressure differentials, driving forces, random events and conditions of surface tension, viscosity, hydraulic head pressure, capillary flow and reversibility, and transient and repairable leaks.'

Harper (1995) discusses the results of various microbial challenge tests cited in the literature. She states that the results of these tests vary depending on test conditions such as channel length, channel diameter, bacterial contaminate concentration, pressure differential, application technique, and fluid viscosity (Harper, 1995).

Maunder et al (1968) found that the biotest organism concentration used for can testing, prior to 1968, of 5 x 10^6 cells/ml was inadequate for testing flexible pouches. This conclusion was based on the fact that 30% of 86 defective pouches failed to show contamination at this level. Therefore, Maunder used a concentration of $20x10^6$ cells/ml for his work with flexible pouches. This research team also noted that maximum incubation time for the test product following the biotest is a function of the interaction of the test organism, packaged product, and temperature (Maunder et al, 1968).

Tallentire and Sinclair (2002) discussed the microbial penetration effect of flow rate with porous materials. With very low flow rates, the extent of spore capture is inversely related to flow rate, so the extent of penetration increases with increasing flow rates (diffusion predominates). The opposite is true of very high flow rates. At higher flow rates, the particles leave the airflow and impact and adhere to the material fibers, resulting in less penetration at higher flow rates (impaction predominates). They suggest a moderate flow rate results in maximum particle penetration, an important measure of the materials biobarrier performance (Tallentire and Sinclair, 2002).

Lampi (1990) concluded, based on physical principles, that microbial penetration would be unlikely to occur in a hole less than 10 microns. The rationale was that in a retort pouch approximately three pounds of pressure are required to overcome capillarity in a 10 micron hole and 3 to 4 psi pressure differential is reasonable for the biotester action. Lampi based his conclusions on a study that involved a 10 micron hole in a retort pouch that was roughly 1/10 the thickness of the pouch material. The test organism diameter was roughly 0.5 micron (Lampi, 1980).

Howard and Duberstein (1980) discovered that certain types of bacterial species naturally occurring in water were able to penetrate 0.2 micron rated filters. They noted a significant retention of the organisms in the filter and that penetration increased over time (Howard and Duberstein, 1980).

Conclusion

As a result of the variation cited in the preceding sections, and in some cases contradiction, of results of cited research, it is clear that biotesting results can be misleading. There is a need to develop a body of knowledge concerning critical hole size for medical device packaging. The research detailed in this dissertation addresses the question of the need for a driving force, other than that introduced by gravity or molecular motion, for a non-porous tray intended for packaging medical devices.

CHAPTER 3

MATERIALS AND METHODS

For reasons detailed in Chapter 1, Introduction, a new method to measure the microbial penetration into packages was needed in order to obtain meaningful results for the primary objective of this research, an examination of the effect of pressure differential on microbial ingress. The new method consists of aseptically filling packages with sterile agar and then incubating closed packages to see if any microbial growth takes place (Appendix I; Figure 22). In order to utilize the new method, it was necessary to verify that the process of filling the package did not significantly influence microbe penetration. Details of this preliminary experiment and results are presented in Appendix I.

Materials

- *Escherichia coli K-12*; ATCC Number 29181

  This organism was examined via scanning electron microscopy (SEM) to measure organism size and to observe morphology. *E-Coli K-12* is a gram-negative, motile, straight-rod that ranges in size from 1.1-1.5μm*2.0-6.0μm. (Brooks et al, 2004) Figure 3 is a photo of the challenge organism used in Experiment One (New Method Development; Appendix I) and Experiment Two (Primary Research).

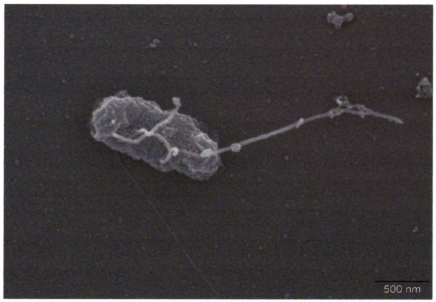


Figure 3 - *Escherichi coli K-12; ATCC 29181*
SEM Photograph

- Trays formed from blue tint, uncoated glycolized polyethylene terphthalate (PETG); nominal preform material thickness; 25 mils. The trays were supplied by Perfecseal from a Medtronic mold; Part Number 350215-001.
- Lidstock-LKF-002 Paper/PE/Foil/PE/HSC Die Cut supplied by Amcor Flexibles

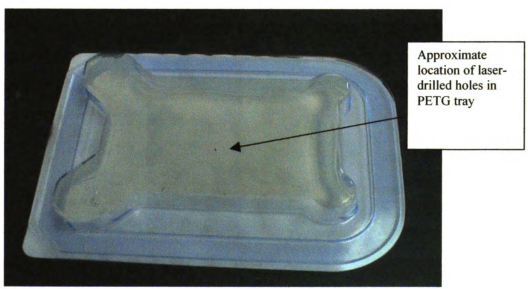


Figure 4 - Test Tray

- Corrugated Shippers and PE bags with ties for transport of trays to the sterilization site
- Nutrient Agar (Difco, a trademark of Becton, Dickinson and Company, San Jose, CA) REF #212000, Lot #2043004
- Nutrient Broth (Difco, a trademark of Becton, Dickinson and Company, San Jose, CA) REF #233000, Lot #3188850
- 60 ml syringes with luer lock tip-Sterile; (Becton, Dickinson and Company, San Jose, CA) REF #309653
- 16-gauge needles, 1.5 inch length-Sterile (Becton, Dickinson and Company, San Jose, CA) REF #305198
- 18-gauge needles, 1.5 inch length-Sterile (Becton, Dickinson and Company, San Jose, CA) REF #305196
- Vented needles with 0.2 micron air filter-Sterile (B. Braun, Bethlehem, PA) REF #40211

Figure 5 - B. Braun Vented Needle System

- Eppendorf tubes with screw cap and O-ring-Sterile (Becton, Dickinson and Company, San Jose, CA)
- 1, 5, and 10 ml pipettes-sterile
- Self Sealing Septum Material supplied by (Mocon, Minneapolis, MN)
- 70 percent Isopropyl Alcohol Swabs
- Non-Latex Exam Gloves
- 50 percent Glycerol Solution
- Chlorine Solution (NaOCl)
- Potassium Phosphate, Monobasic, Crystal (KH_2PO_4)
- Becton Dickinson Enterotube™ II, Catalog #211832, for the identification of gram negative bacteria. (Becton, Dickinson and Company, San Jose, CA)

Services

- Gamma Sterilization - The appropriate dose, in kiloGrays, was determined in conjunction with the gamma sterilizer provider. The dose level and process records were recorded. Sterigenics, the radiation sterilization provider, indicates that PETG has a 1000 kiloGray tolerance level and the material remains very stable and maintains clarity (www.sterigenics.com, 2005).
- Laser Hole Drilling - Thermal laser drilled holes were placed in the same location on each test tray; the formed PETG tray in the center of the bottom of the tray (Figure 8). The laser holes were drilled by Lenox Laser, Glen Arm, MD. The holes drilled were 10 micron ± 10% and 100 micron ± 5% in diameter. The hole diameter and flow rate were provided by Lenox Laser for each test tray. (Reference Results Tables 15, 16, 17, and 18)

Equipment

- Aerosolization Cabinet

  The test trays were sprayed with *E. coli K-12* in a closed, airtight, Plexiglas® cabinet. The internal dimensions of the cabinet are 28.5 in x 20 in x 36 in (LxWxH). The Plexiglass® is 0.25 inch thick. The aerosol containing the microorganisms was delivered through dual pressurized (40 psi) spray canisters mounted on the top of the cabinet, the aerosol remained within the cabinet. The canisters were Central Pneumatic Air Spray Guns Model #43760 with an external mix nozzle 0.082 inches in diameter. The cabinet has an integrated racking system designed to hold four test trays and syringes in a uniform orientation for testing. The rack surface is 12 inches from the floor of the cabinet. The cabinet has two gloves integrated into the front wall (Reference Figure 7), enabling sample and syringe manipulation while the cabinet is sealed.

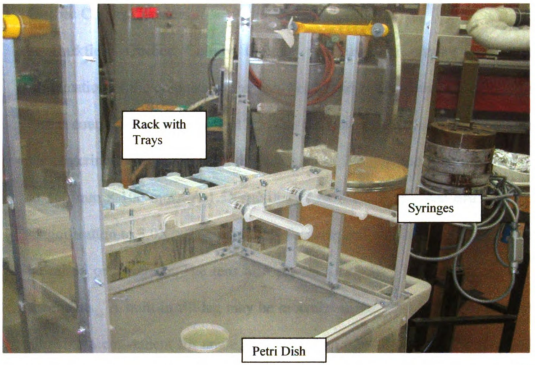


Figure 6 - Racking System Inside Aerosol Cabinet

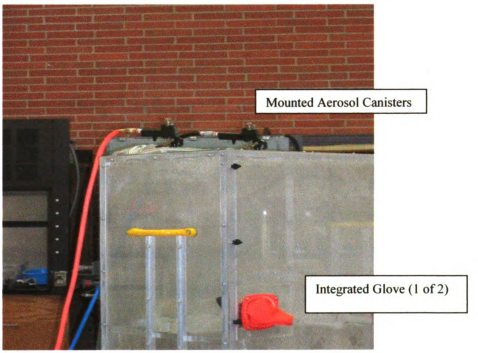


Figure 7 - Mounted Canisters on Aerosol Cabinet

- Quebec Colony Counter

  A Darkfield Quebec Colony Counter was used to provide even, glare-free illumination of the colonies so they are bright and easily distinguished to allow for a visual count (Reference Figure 17). The counter has an adjustable specimen holder for centering round dishes with diameters up to 100 mm and square culture plates up to 100 mm x 100 mm. A 1.5X auxiliary lens fits over the standard lens increasing magnification to 3.0X. The adjustable focusing rod allows the 1.5X standard lens to be raised or lowered. The lens also rotates a full 360° for ready access to culture specimens. A built-in tilt leg may be mounted in the front or rear of the instrument allowing a convenient tilt angle, or it may be locked flat to the instrument base. A white-ruled Wolffheugel counting plate was used to facilitate the counting of colonies

  .

- Heat Sealer

  SenCorp Tray Sealer, Series MD2420 (SenCorp, Hyannis, MA), dual-shuttle heat sealer with tray 9-up tooling - Tray die position was recorded.

Methods

Outlined below are procedures A though K. These procedures were developed specifically for the research presented in this dissertation. Procedure A, 'Microbial Cabinet Analysis' was run first, followed by Procedure B, 'Experiment Two Procedures.' Procedures C through K support and are referenced in Procedures A and B.

All microbial and medium handling procedures were developed and conducted in accordance with 'Current Protocols in Molecular Biology'(2005).

Procedure A - Microbial Cabinet Analysis

The development of a microbiological growth curve was performed as described here. It was intended to illustrate that:

- The organisms in the aerosolized state were viable and to quantify viability.
- The dosage level of microbes delivered during aerosolization.
- The consistency of the aerosol within the cabinet.
- The growth rate of *E. coli.*
- No other contaminants are present within the system that can survive on the medium in the trays.
- An absence of contaminants after disinfecting the cabinet.

1. To determine the dosage of microbes delivered during aerosolization and the duration of aerosolization, the aerosol system was run with deionized water in an empty, clean cabinet. The two aerosol canisters were each filled with 750 ml deionized water as measured with a graduated cylinder. The set up was the same as intended for the primary experiment.
2. The time that elapsed until the cabinet floor was covered with a 'film' of liquid was recorded as 15 seconds. A 'film' is defined as a complete, visible covering of the surface with no pooling. The remaining volume in the reservoir was measured as 725 ml for each canister. Therefore, the total volume delivered was 50 ml. This duration, 15 seconds, was used as the starting duration for the development of a growth curve.
3. A total of 28 sterilized trays (8 trays per microbial concentration and 4 for controls) sealed with non-porous lidstock were tested (per Materials Section).

Gamma sterilization was provided by Smith & Nephew Orthopedics and performed at Sterigenics. The Run Number was SMN01 SNN 27050270; run date 11/22/05. The run was performed per Smith & Nephew process procedure P000257-L. A minimum dose of 26.5 kGy and a maximum dose of 40.9 kGy was recorded.

4. Using aseptic technique (steps 1-7 of Figure 22), a minimum of 25 ml of sterile medium was added through the self-sealing septum material using a sterile syringe and needle as described in Procedure H "Addition of Medium to Trays". Difco Nutrient Agar Manufacture Number 212000; Lot Number 2043004 was used.
5. Once the medium solidified, the lidstock was removed from the tray (4 at a time) and the trays were placed bottom-side down on the rack inside the cabinet, Reference Figure 6. The trays were identified as '1' through '4.' The tray orientation was consistent from run to run. Trays and dishes were positioned as follows (Figure 8):

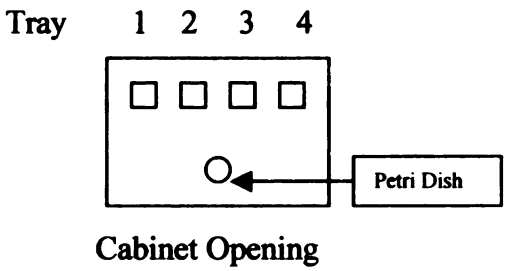


Figure 8- Cabinet Test Configuration-Top View

6. Also placed on the floor of the cabinet (uncovered) was a sterile petri dish containing the same agar as the trays. The dish remained covered until use. The removed lidstock and covers were handled and stored in such a manner as to

minimize contamination because they were to be used again as covers for the test units during incubation. The cabinet was closed and the aerosolization process begun.

7. Each group was run separately. The first group was run with 10^2 CFU/mL microbe concentration, the next 10^4, with the final concentration of 10^6 (per dilution chart in Procedure I, "Microbial Aerosolization Procedure").
8. Since the groups were run in ascending order with regard to microbial concentration, cleaning was not performed until the completion of all test groups.
9. Beginning with a microbe concentration of 10^2 (per dilution chart in Procedure I) the aerosol process was run for the duration of 15 seconds as determined in Step 2 of this procedure. The trays and the petri dish remained in the cabinet for 30 minutes (including aerosolization time), then were moved to the environmental chamber.
10. The original lidstock was placed over the opening of the tray and the cover was returned to the petri dish.
11. The trays and dishes were placed in an environmental chamber set at 37°C +/- 2°C; 50% RH for 36 hours. Observable colony forming units were quantified using a Quebec Colony Counter per Procedure J, "Enumeration of Colonies". Time intervals were originally set at 12 hours, 18 hours, 24 hours, 48 hours, and 72 hours because results from Experiment 1 (Appendix I) indicated that evidence of growth after 12 hours and the agar began drying after 72 hours. The actual time intervals were 12 hours, 24 hours, and 36 hours. The 18-hour interval was eliminated due to schedule constraints and the 48 and 72 hour eliminated due to

colony saturation and agar drying. The agar drying earlier than noted in Experiment 1 was likely due to the loose cover of the removed lidstock allowing for increased air circulation.

12. Steps 8 through 10 of this procedure were repeated using an aerosol exposure duration of 30 seconds. The remaining volume in the canisters for the doubled time was 700 ml, resulting in a total volume delivered of 100 ml.
13. Steps 5 through 12 of this procedure were repeated for microbial concentrations of 10^4 and 10^6.
14. The cell growth versus exposure time for each concentration was plotted. The amount of growth on the petri dish was recorded and was used for comparison to the dishes run in Experiment 2. The data that follows is from the 36-hour time interval as it was the maximum growth interval before the agar began to deteriorate. The results for the 10^6 concentration delivered for 30 seconds were difficult to quantify because after the 12-hour point the CFUs became too numerous to quantify with great accuracy. Therefore, the slope of this concentration is not as meaningful as the other concentrations. The key data point for this concentration was approximately 100 CFU/sqin and was noted at the 15 second aerosol duration.

Table 7
Colony Forming Unit Quantification From Growth Curve Study (Units CFU/sqin)

10^2 15 seconds	*Tray 1*	*Tray 2*	*Tray 3*	*Tray 4*	*AVG*	*Plate*
12 hour	3	8	14	17	11	4
24 hour	8	27	21	27	21	12
36 hour	22	40	32	46	35	30

10^4 15 seconds	*Tray 1*	*Tray 2*	*Tray 3*	*Tray 4*	*AVG*	*Plate*
12 hour	22	22	31	42	29	25
24 hour	48	46	59	65	55	47
36 hour	72	75	73	84	76	68

10^6 15 seconds	*Tray 1*	*Tray 2*	*Tray 3*	*Tray 4*	*AVG*	*Plate*
12 hour	58	62	72	52	61	54
24 hour	72	71	86	77	77	72
36 hour	114	104	99	88	101	97

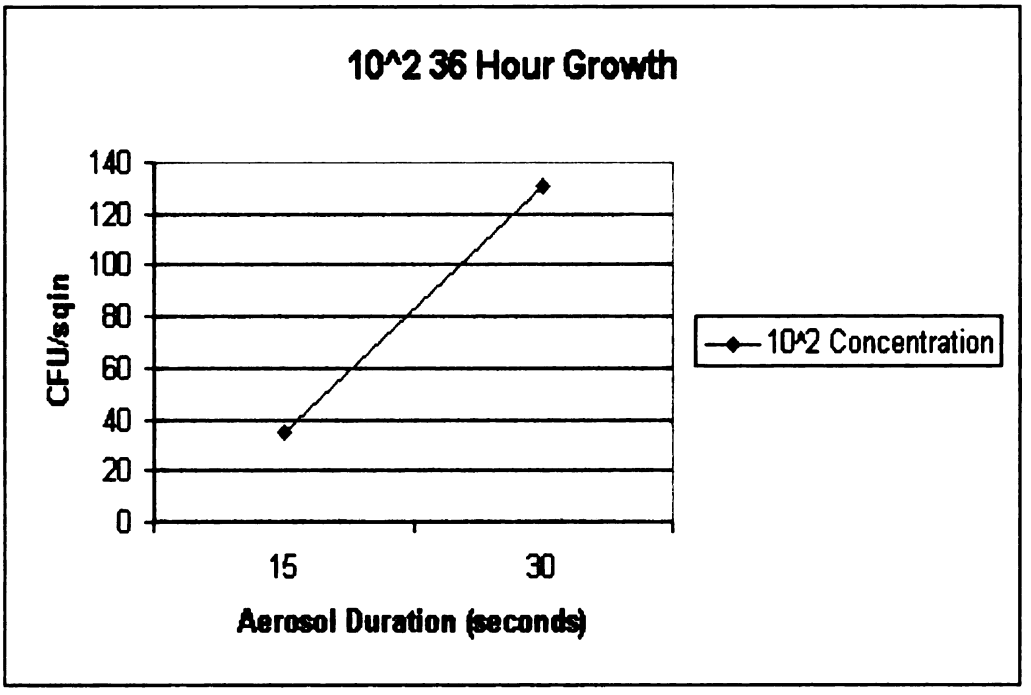
10^2 30 seconds	*Tray 1*	*Tray 2*	*Tray 3*	*Tray 4*	*AVG*	*Plate*
12 hour	22	38	34	21	29	24
24 hour	68	77	66	64	69	67
36 hour	119	144	140	121	131	121

10^4 30 seconds	*Tray 1*	*Tray 2*	*Tray 3*	*Tray 4*	*AVG*	*Plate*
12 hour	66	61	52	57	59	54
24 hour	108	85	93	119	101	87
36 hour	171	153	157	164	161	151

10^6 30 seconds	*Tray 1*	*Tray 2*	*Tray 3*	*Tray 4*	*AVG*	*Plate*
12 hour	89	105	107	106	102	97
24 hour	250	250	250	250	250	250
36 hour	250	250	250	250	250	250

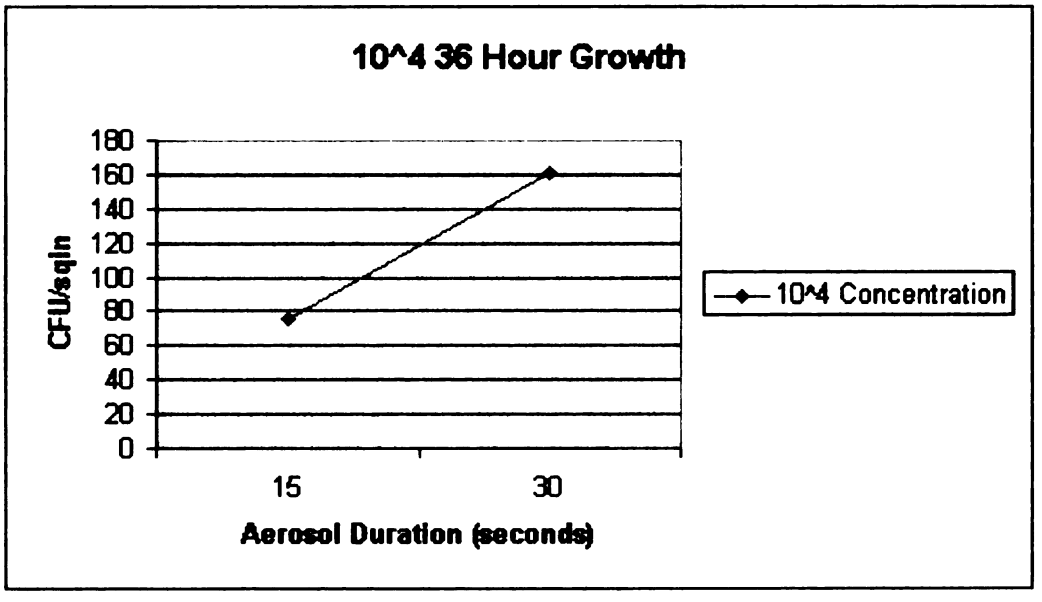
Following Disinfection-30 second delivery of sterile buffer solution

	Tray 1	*Tray 2*	*Tray 3*	*Tray 4*	*AVG*	*Plate*
12 hour	0	0	0	0	0	0
24 hour	0	0	0	0	0	0
36 hour	0	0	0	0	0	0



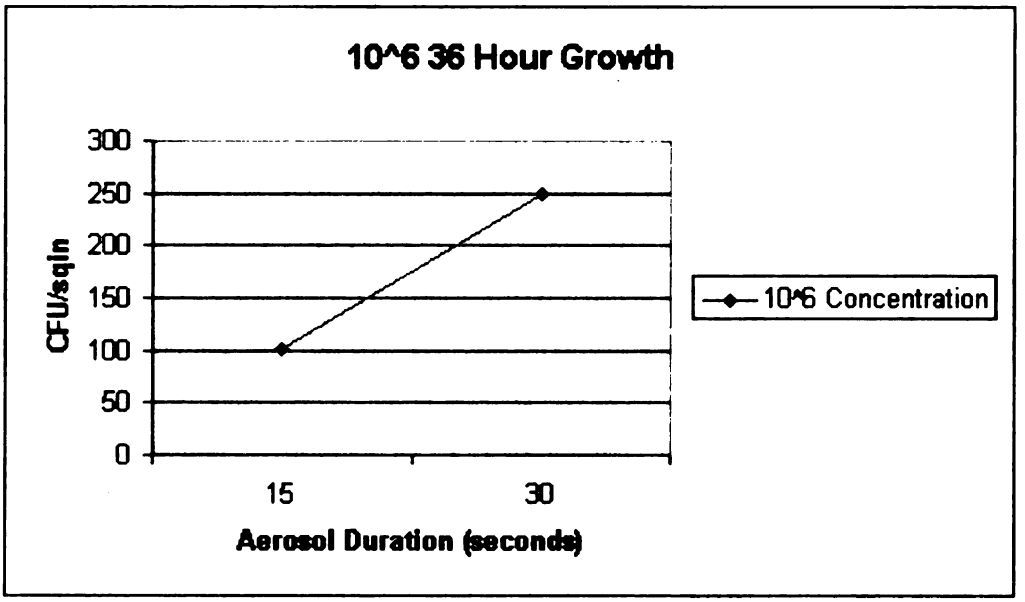
y=mx+b
y=6.33334x + (-60)
Duration resulting in 100 CFU/sqin is 100=6.33334x-60=**25.2 seconds**

Figure 9 - 10^2 Concentration 36 Hour Growth



y=mx+b
y=5.666667x + (-10)
Duration resulting in 100 CFU/sqin is 100=5.666667x-10=**19.4 seconds**

Figure 10 - 10^4 Concentration 36 Hour Growth



y=mx+b
y=10x + (-50)
Duration resulting in 100 CFU/sqin is 15 seconds

Figure 11 - 10^6 Concentration 36 Hour Growth

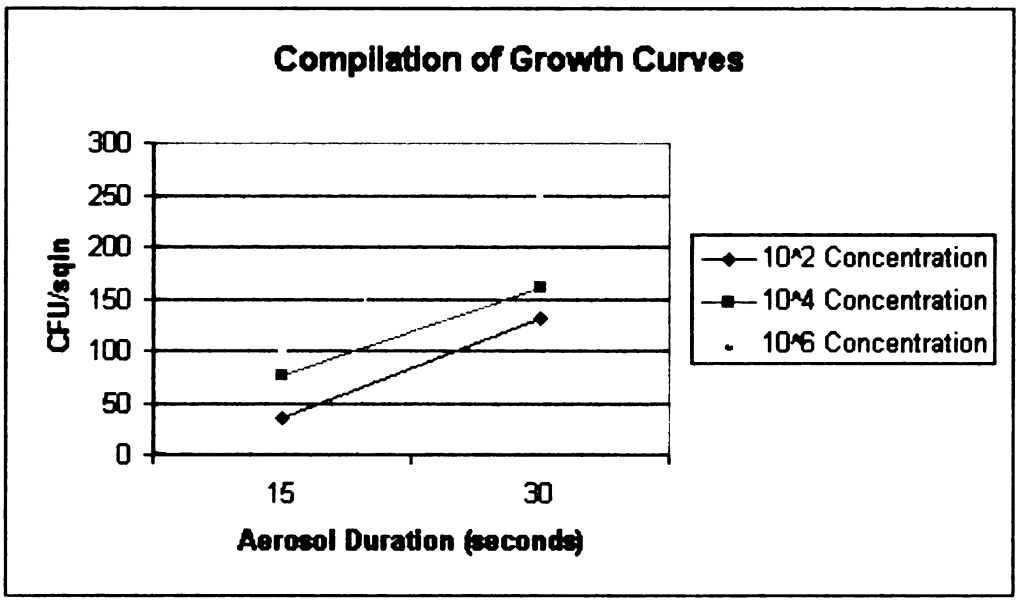


Figure 12 – Compilation of Growth Curves for 3 Microbe Concentrations

15. The volume delivered for each microbe concentration that yields approximately 100 CFU/in^2 of media was determined, Reference Figures 9, 10, and 11. The time to deliver that volume was determined. This became the aerosol duration time for the remaining experiments. Per the above graphs, the times which yield approximately 100 CFU/in^2 of media, are:

    25 seconds of aerosolization when using a concentration of 10^2

    19 seconds of aerosolization when using a concentration of 10^4

    15 seconds of aerosolization when using a concentration of 10^6

16. For the duration time selected for the experiments (Step 14), the 'Dose Delivered' column in the following table was completed with the volume times the concentration (cells/ml), and the 'Viable Organisms' column with the average number of viable organisms on the tray per square inch (CFU/in^2). This gives an indication of the loss of microbes to the aerosol process.

Table 8-Microbial Dose Delivery

Canister Volume	Microbe Dilution Volume	Resultant Concentration	Dose Delivered	Viable Organisms
1500 ml (750 x 2)	1.5 ml of 10^9 cells/ml	1 x 10^6 cells/ml	50 x 10^6 cells/ml	101/sqin
1500 ml (750 x 2)	1.5 ml of 10^7 cells/ml	1 x 10^4 cells/ml	63 x 10^4 cells/ml	100/sqin
1500 ml (750 x 2)	1.5 ml of 10^5 cells/ml	1 x 10^2 cells/ml	83 x 10^2 cells/ml	100/sqin
1500 ml (750 x 2)	No addition	0	50 ml (sterile buffer solution)	0/sqin

17. A final group intended to illustrate the 'absence of microbes' was repeated after the prescribed disinfection process using 2500ppm chlorine solution (NaOCl) to wipe down the cabinet and autoclave the canisters and spray apparatus.
18. 0 CFU/mL microbe dilution (Sterile Butterfield's Buffer Solution) was aerosolized for the 30 seconds on 4 trays and a petri dish placed as in Step 5 of this procedure.
19. The trays were incubated and any growth counted as in Step 10 of this procedure. Results are included in Data Table in Step 14 (Table 7).
20. The results were reviewed for verification of functionality of the cabinet and aerosol system, viability of the aerosolized *E. coli*, efficacy of the disinfection process, and adequacy of the prescribed time intervals for quantifying growth of the *E. coli K-12* to be used for this research.

Discussion-Procedure A

The generation of the microbiological growth curve for the newly constructed aerosolization cabinet was performed successfully. The following was illustrated:

1. The organisms in the aerosolized state are viable and the viability was quantifiable. *Reference data in Step 14 (Table 7).*
2. The dosage of microbes delivered during aerosolization was determined. *Reference data in Step 16 (Table 8).*
3. The consistency of the aerosol within the cabinet. The data indicates an observed consistent distribution of aerosol on the test trays. In Experiment Two the trays were rotated for each run to minimize the impact of positional variation.

Figure 13 illustrates the consistent distribution of microbes on a petri dish from the study. This is representative of growth noted on test trays.

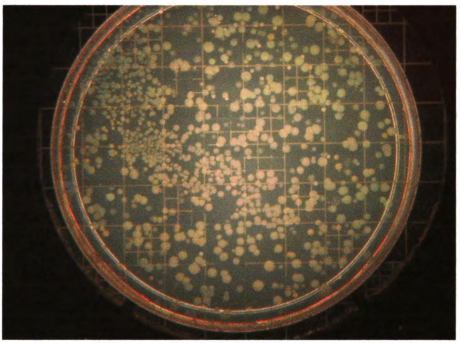


Figure 13 - Petri Dish Exhibiting Microbial Growth

4. The growth rate of E. coli. *Reference data in Step 14 (Table 7).*
5. No other contaminants were present in the system that survived on the medium in the trays. No particles or foreign colonies were noted during observation.
6. An absence of contaminants after disinfecting the cabinet. Following the disinfection process no growth was noted on the test trays or the Petri dish. *Reference data in Step 14 (Table 7).*

Procedure B – Experiment 2 Procedures

1. A total of 400 PETG trays and 400 die-cut lids were obtained.

2. The trays were packaged in double polyethylene bags. The bags were tied and the trays were then placed in a corrugated case for transport to the laser hole drilling provider, Lenox Laser in Glen Arm, Maryland.
3. Lenox Laser drilled a 100 micron hole ± 5% in 200 PETG trays and a 10 micron hole ± 10% in 200 PETG trays. Each hole was placed in the center of the tray bottom. The hole area was circled with black marker. Each tray was also labeled with a pressure sensitive label carrying a tray number, hole size, and hole flow rate in standard cubic centimeters. Reference Figure 14 for an image of the label and reference the Results Section, Chapter 5, for the raw data.
4. Following the thermal laser drilling of holes in the tray, the trays were then returned to Michigan State University.
5. The packages were sealed on the SenCorp MD 2420 dual shuttle tray sealer. Sealer die position was recorded. Sealing parameters were set at the following: Seal temperature – 325°F, Seal Duration – 2.5 seconds, and Seal Pressure at 70 psi. The trays and seals were visually inspected for defects in the tray and lidstock. Seal adequacy was visually verified per ASTM F1886 'Visual Tests, destructive and non-destructive.' Any trays detected to have defects were removed from the study.
6. The trays were again packaged in double polyethylene bags. The bags were tied and the trays were then placed in a corrugated case for transport to the gamma sterilization facility in Memphis, Tennessee. Gamma sterilization was provided by Smith & Nephew Orthopaedics and performed at Sterigenics. The Run Number was SMN01 SNN 27050270; run date 11/22/05. The run was performed

per Smith & Nephew process procedure P000257-L. A minimum dose of 26.5 kGy and a maximum dose of 40.9 kGy was recorded.

7. Following the sterilization, the trays were returned to Michigan State University.
8. The trays remained in the cases until the addition of media.
9. Care was taken to avoid any contamination of test trays while handling. A sampling of trays was examined visually as in Step 2 of this procedure to ensure the integrity of the trays. Again, trays determined to have damage were discarded.
10. Medium was added to the trays as described in Procedure H.
11. The experiment was conducted in the test group order described in Chapter 4, Experimental Design. For each group the trays were placed in an inverted position in a racking system designed to secure the trays and syringes in a uniform configuration. Reference Figures 6 and 8 for a depiction of test tray configuration.
12. For the treatments that required the pressure differential, a self-sealing septum was placed on the end of the tray. Prior to application to the tray, each septum were immersed in 70 percent isopropyl alcohol and wafted though a flame to remove any excess alcohol. The trays that were not exposed to a pressure differential did not require the addition of the septum material. All trays were oriented identically.
13. A sterile 16-gauge needle was placed on a sterile 60 ml syringe. The septum was pierced aseptically and the needle/syringe system was secured in the cabinet

racking system. The bracket was then placed over the end of the syringe plungers, reference Figure 6.

14. An open petri dish, containing the same agar used in the trays, was placed on the bottom of the cabinet. The placement of the petri dish was the same as that depicted in Procedure A (Figure 8). The petri dish was sterile and remained covered until placement into the cabinet. As mentioned, the petri dishes served as an indicator of the viability of the organisms and the dose delivered when compared to the plates produced in Procedure A.
15. Immediately following the sealing of the cabinet, Procedure I was initiated; Microbial Aerosolization, at the appropriate test group concentration.
16. After the aerosol had run for 15 seconds, the prescribed duration, the bracket was retracted, simultaneously withdrawing 62 ml of air volume from each of the test trays for which a pressure differential was induced. The rate of retraction was consistently one minute per plunger retraction to 62 ml mark (reference 'Pressure Differential' section in Experimental Design section for a discussion concerning rate). This was accomplished following the aerosolization process, without opening the cabinet, using gloves integrated into the front cabinet wall (see Figure 7).
17. Upon completion of the cycle (aerosolization duration and time in cabinet = 30 minutes), the cabinet was opened and the trays removed from the rack. The needles and syringes were removed from the trays prior to placing them in the incubator.

18. Trays were placed, lidstock down, in an environmental chamber controlled at 37°C +/- 2°C; 50% RH for 72 hours. Observable colony forming units were quantified by using a Quebec Colony Counter per Procedure J. Inspection time intervals were 12 hours, 18 hours, 24 hours, 48 hours, and 72 hours. The only quantification recorded was that of the last inspection time, 72 hours, as this was the only time the lidstock was removed from the tray to obtain a full view of the growth media. The pressure sensitive label placed on the tray bottom by the laser provider identifying the hole size and flow rate restricted full view of the media. Reference Figure 14.
19. To verify that CFUs observed were the test organism, *E. coli K-12,* the colonies were sampled and cultured in a Becton Dickinson Enterotube™ II System, a multimedia tube intended to perform as an identification system for specific member organisms of the family of *Enterobacteriaceae.* Reference Procedure K.

Procedure C - Preparation of Sterile Media

An adequate number of Erlenmeyer Pyrex Glass Flasks for media containment were obtained. Care was taken when selecting flask volumes to not have media more than 1 inch from any container surface for adequacy of sterilization.

1. Flasks were clean and free of any debris. Prior to use, they were rinsed thoroughly with deionized water (DW).
2. An appropriate volume of DW was measured with a graduated cylinder and transferred to the flask.
3. Using a calibrated scale, the appropriate amount of nutrient agar powder was measured and added to the flask containing DW. Care was taken throughout the

operation to not introduce any contaminants. The flask was gently swirled to mix. Agar used for this experiment was Difco Nutrient Agar; REF #212000, Lot#2043004. The dilution was 23g per 1000 ml of DW.

4. A minimum of 30 ml of agar solution was transferred to a 100 ml test tube.
5. A foam stopper of adequate size was placed in the opening of the tube with the liquid media. The stoppers fit tightly. Aluminum foil was placed over the stopper and top of tube.
6. A small piece of autoclave indicator tape was placed on the foil. Care was taken to not put adhesive in direct contact with glass.
7. The filled test tubes were then ready to be autoclaved.
8. Prior to autoclaving, the water bath was brought to the correct temperature; 47^0C +/- 2^0C
9. The tubes were placed in a tube rack and placed directly on the bottom surface of the autoclave chamber, centering as much as possible.
10. The chamber door was then closed.
11. Cycle time was set to 25 Sterilize (minutes); Dry (minutes) 0.
12. The cycle was started by pushing the 'on' button and 'fluid' button.
13. The cycle begins when conditions reach 121^oC and 15psi.
14. When complete, care was taken to stand away from the chamber opening as the door was opened.
15. The door was then slowly opened to allow for steam release.
16. Using asbestos gloves, product was removed and transferred to the water bath.

17. The water bath level covers the 'height' of agar in the tubes, but care was taken to ensure that it was below the level of the top of the test tubes. Too low of a level has the potential to result in gelling of solution, too high a level can result in surface condensation, a possible contaminate.
18. Agar was used within 6 hours after preparation. To ensure agar sterility, a flask of the each sterilized agar lot was incubated at 37°C while being gently agitated in flask shakers for 24 hours. The samples did not exhibit microbial growth indicating adequacy of the sterilization process.

Procedure D - Preparation of Microbial Stock Solution

1. Two 100ml lots of Difco Nutrient Broth; Product 233000, Lot #3188850, were prepared in two, 250 ml Erlenmeyer flasks.
2. The lots were autoclaved for 25 minutes at 121°C as described in *Procedure C.*
3. One ml of sterile nutrient broth was added to the lyophilized *Escherichia coli K-12* vial; ATCC 29181, using a sterile pipette.
4. The vial was agitated ensuring that reconstitution was complete.
5. 0.5 ml of reconstituted solution was transferred, using a sterile pipette, to each 250 ml Erlenmeyer flask containing 100 mls of sterile nutrient broth.
6. Solutions were incubated at 37°C for 24 hours. Flasks were gently agitated during the incubation period.
7. 200 Eppendorf Polypropylene tubes with O-Ring Screw Caps were autoclaved for 15 minutes at 121°C.
8. 100ml of 50% Glycerol Solution was autoclaved. This served as a cryoprotective agent for the microbial stock solution.

9. 100 Eppendorf Polypropylene tubes were prepared from each 250ml Erlenmeyer Flask. Each tube contained 1ml of microbial solution and 0.4mls of 50% Glycerol. The transfer was performed with sterile pipettes using aseptic technique.
10. One tube from each 'lot' was used to determine the starting microbial load of the stock solution via the growth curve method. Starting Load for this *E. coli* stock solution=$1x10^9$ CFU/mL.
11. The remaining tubes from each 'lot' were adequately labeled and placed in a polystyrene sleeve and stored at –80°C until use.

Procedure E - Preparation of Microbe Dilutions

1. *E. Coli* stock solution was removed from –80°C freezer and placed in wet ice until use.
2. When ready to use, the vial was manipulated by hand to melt. Once melted, inverted to mix (approximately 10 times).
3. Using a sterile 1ml pipette, 1ml was drawn from the Eppendorf tube. The ml was placed in 100 mls of sterile Butterfield's Solution produced per Procedure G.
4. The dilution was agitated vigorously for a minimum of 30 seconds to ensure an equal distribution of cells as well as to break up clumps of organisms into individual cells. This gives a 10^{-2} microbe dilution.
5. Once adequately mixed, 1ml of the 10^{-2} dilution was withdrawn in a sterile pipette. The ml was added to a fresh 100mls of Butterfield's Solution. Manipulation was repeated. This gives a 10^{-4} dilution.

6. Further dilutions were made by transferring 1 ml aliquots into additional dilution blanks. The process was repeated to obtain 10^{-6} and 10^{-8} dilutions.

Procedure F - Preparation of Plates with Microbe Dilutions/Pour Method

1. The dilutions prepared in Procedure E were agitated vigorously for a minimum of 30 seconds.
2. 1 ml aliquots of a dilution were added to a sterile petri dish.
3. Sterile liquid nutrient agar was added to the plate enough to fully cover the bottom of the dish.
4. The dish was gently swirled to mix the agar and the microbe dilution
5. A cover was placed on the dish.
6. The plates were incubated at 32°C for a minimum of 24 hours.
7. Using a Quebec Colony Counter the colonies on the plate were counted.

   To determine the Standard Plate Count (SPC) of the dilutions: SPC=diluted plate count/dilution ratio

Procedure G - Preparation of Butterfield's Buffer Solution

1. Stock Solution contains:

   Potassium Phosphate, Monobasic, Crystal (KH_2PO_4) 34g J.T. Baker CAS 7778-77-0 Distilled Water 500ml
2. After preparation of the stock solution, pH was adjusted to 7.2 with 1 N Sodium Hydroxide (NaOH). The volume was brought to 1 liter with distilled water.
3. The solution was autoclaved 15 minutes at 121°C and stored in refrigerator (4°C) until use.

Procedure H - Addition of Medium to Trays (Reference Figure 22)

1. Sealed, sterile empty trays were obtained.
2. Test tubes containing 30 mls of medium (1 Test Tube per Test Tray) were autoclaved. Cycle time was set to 25 Sterilize (minutes); Dry (minutes) 0.
3. The tubes of agar were equilibrated in water bath to 47^0C +/- 2^0C.
4. Each tray was uniquely coded.
5. With a 70 percent Isopropyl Alcohol swab, an approximately 2 sqcm area on the center of the tray lidstock was swabbed using moderate pressure for approximately 30 seconds.
6. A pre-sized (approximately 2 cm^2) piece of septum material was picked up using disinfected forceps.
7. The backing material was removed with forceps and discarded, exposing the adhesive material.
8. The septum material was dipped in a 70 percent isopropyl alcohol bath.
9. The disinfected septum was wafted in the flame of a Bunsen burner to flash off any remaining alcohol.
10. Still using forceps, the septum material was placed, adhesive side down in the center of the disinfected area of the tray.
11. The septum material was firmly pressed with the forceps to ensure adhesion.
12. The trays were placed in the jig designed to orient the trays lid-side down while adding medium and providing a consistent point and depth of needle entry, reference Figures 14 and 16.

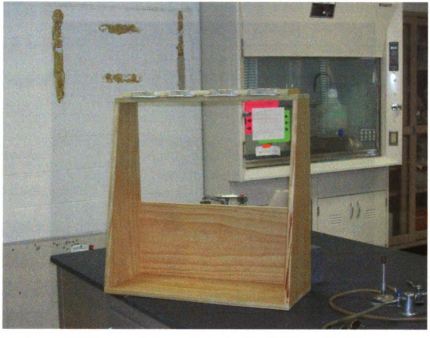


Figure 14 - Jig Designed for Uniform Introduction of Media to Test Trays

Figure 15 - Top View of Test Trays Loaded in Jig

Figure 16 - Bottom View of Trays Loaded in Jig

13. The test tube stopper was swabbed with a 70% isopropyl alcohol swab for approximately 30 seconds.
14. Approximately 25 mls of equilibrated medium was drawn up aseptically in a sterile syringe using a 16-gauge needle from the test tube. The tube remains closed during this process and the needle pierces the tube stopper.
15. The 16-gauge needle was replaced with a sterile vented needle. The use of a vented needle allows for out gassing as the medium is injected into the non porous package.
16. With a 70 percent Isopropyl Alcohol swab, the septum material was swabbed using moderate pressure for approximately 30 seconds.
17. The entire syringe volume was injected into the tray through the septum through the fixture in the jig, reference Figure 16.
18. The tray was agitated to ensure an even distribution of agar.

19. The medium was allowed to completely solidify undisturbed.
20. If more than 3 minutes elapsed after a disinfecting procedure before the next operation was performed, the disinfecting procedure was repeated.
21. This process was repeated for each test tray.

Procedure I - Microbial Aerosolization Procedure

1. Each aerosol canister was filled with 750 ml of sterile Butterfield's Buffer Solution. The solution was then inoculated with 0.75 ml of the desired concentration of *E. coli* per the table below:

Table 9-Microbe Dilution Volume/Resultant Concentration

Canister Volume	Microbe Dilution Volume	Resultant Concentration
750ml x 2=1500ml	0.75 ml of 10^9 cells/ml per canister	1 x 10^6 cells/ml
750ml x 2=1500ml	No addition	0

2. Both pumps are then turned on and the test trays sprayed for the duration determined in Procedure A (15 seconds).
3. After the cycle, test trays designated for exposure to a negative pressure differential, have syringes simultaneously drawn back via the cabinet racking system. The trays and syringes remain in this position for 30 minutes (total cycle time).
4. Following the complete cycle, the trays are removed from the chamber; syringes and needles are removed and the trays are placed in the appropriate environmental chamber. The hole created by the needle is covered upon needle withdrawal by

the self-sealing septum material placed on the tray before the needle puncture was made.

5. Following test completion, the cabinet is wiped with chlorine bleach and the canisters rinsed with bleach and autoclaved.

Procedure J -Enumeration of Colonies

1. The tray, PETG side facing out, was placed on the Quebec Colony Counter for illumination.
2. To facilitate the count, a water-soluble marker was used to identify colonies counted. If a marker was used care was taken to avoid the laser drilled hole. The magnifying glass was used if needed.
3. The number of colony forming units (CFU) was recorded for each time interval for each tray.
4. If the tray exhibited too much growth to accurately identify individual CFUs, this was recorded as 'too numerous to count' or TNTC.
5. Any suspicious contamination observed was noted and removed from the tray media after the final time interval, and cultured on selective media for the organism being tested to determine if it was *E. coli*. Microscopy was also used to investigate the source of any unidentified contaminants.

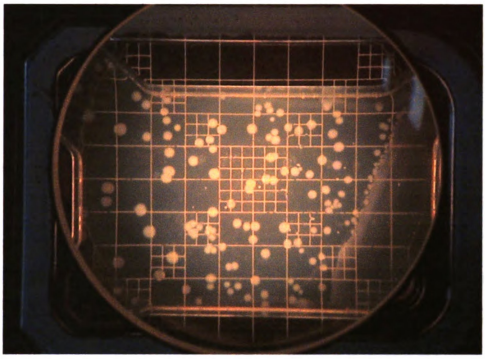


Figure 17 - Tray Exhibiting Growth on Quebec Colony Counter

Procedure K - Identification of gram negative colonies via the Enterotube™ II

1. All CFUs observed on test trays following incubation are prepared to be cultured with this system for verification as the test organism, *E. coli K-12.*
2. Remove both caps from the tube ends.
3. Place the tube inoculating wire into the well isolated colony, avoid touching agar.
4. Inoculate the tube by inserting the wire through the twelve tube compartments and then withdrawing using a turning motion.
5. Stop wire withdrawal when the notch on the wire is aligned with the tube opening.
6. Break the wire at the notch by bending.

7. Punch holes with the broken piece of the wire covering the air inlets of the last eight compartments in order to support aerobic growth in these compartments.
8. Replace both caps to the tube.
9. Incubate at 37°C for 18 to 24 hours.
10. Remove from incubation and interpret the results visually per the results pad provided with the system.
11. Following recording of the results, perform the indole test by adding via syringe and needle two drops of Kovacs' reagent through the plastic film of the H_2S/indole compartment. A positive reaction is noted by development of a red color in the added reagent on surface media within 10 seconds. This result is added to the results pad for interpretation. Kovacs reagent consists of :

    Amyl or isoamyl alcohol.....75 ml

    p-Dimethylaminobenzaldehyde.....5 g

    hydrochloric acid concentrated.....25 ml

12. The ID value arrived at on the provided results pad is located in the provided interpretation guide. The corresponding organism is reported.

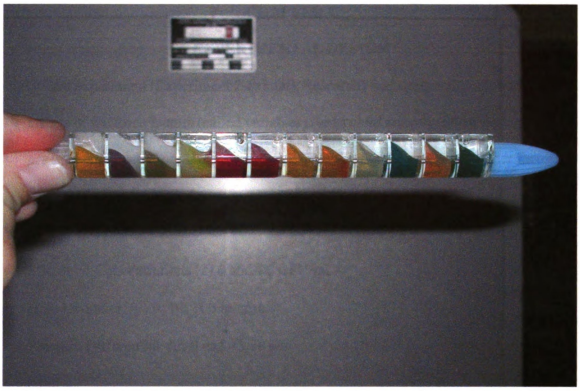


Figure 18 - B-D Enterotube™II – Identification System for *Enterobacteriaceae*

CHAPTER 4

EXPERIMENTAL DESIGN

An experiment (Experiment 2-Primary Research) was designed to estimate the effects of the three factors (called variables): *microbial concentration*, *hole size* and *pressure differential* on the ingress of *E. Coli K-12* into the test trays. Other factors were held constant (called constants).

Variables

- Microbial Concentrations (2) 0 and 10^6CFU/mL
- Hole Diameter (3) 0, 10, 100 micron
- Pressure Differential (2) 0 and –3.78 psi

Constants

- Tray and Hole Orientation and Tray Geometry
- Tray Materials
- Microbe; Motility
- Aerosolization Method
- Microbial Exposure Time
- Temperature

Response Variable

- Colony counts of *E. coli K-12* in the test tray

Justification of Design

It is understood that a variety of factors relating to the package have the potential to influence microbial penetration (as previously outlined), but it is believed that keeping the design of this experiment as simple as possible is the best approach, and will provide

information that will be useful to build future research that incorporates more of these variables. Following are the details regarding the variables and the constants in this experiment.

Variables Details

- Microbial Concentrations (2) 0 and 10^6CFU/mL

  The results from Experiment 1, detailed in Appendix I, indicate the contamination threshold lies between a concentration of 10^4 CFU/mL and 10^6 CFU/mL.

  The theoretical exposure of the test trays is 2.5 x 10^6 cells per cubic meter. This is based on the maximum microbe concentration used in this study. An average hospital has an airborne microbe level of 125 cells per cubic meter (Schmidt et al., 1984). Based on this estimate, approximately 20,000 times this load will be evaluated at the 10^6 being employed in this study. In a controlled medical device manufacturing area the airborne microbial load would be expected to be significantly less than that of a hospital (Reich, 1985) or other end use environments.

  A greater bio-burden than these packages would typically face was chosen for several reasons. Anema et al (1980) indicated that when using a biotest to quantify the amount of leakers, the concentration of organisms should be relatively high to ensure that all leakers are infected. Put (1972) suggested that, based on her work with canned foods, fewer leakers will be detected when microbial loads are lowered. Testing labs currently conducting microbial aerosolization testing for various medical device manufacturers indicate they routinely begin with a microbial concentration of 10^7 or 10^8 cells, resulting in product exposure of 10^4 and 10^5 cells respectively (Mycoscience, Inc, Wilmington, CT and AppTec, Inc, St. Paul, MN). Additionally,

the higher burden presents a more hostile challenge than packages face in normal distribution environments, providing a 'safety factor' for published results.

- Hole Diameter 0 (no hole), 10, 100 micron

  0 (no hole) is a negative control.

  Reference Figure 14 for a tray photo identifying approximate hole location.

  Ten and 100 micron holes were drilled in 200 test trays of each hole size by Lenox Laser (Glenn Arm, MD) using a thermal laser. Ten microns was selected as the defect size to be tested, as it is representative of what previous research has documented as critical (Blakistone et al, 1996; Harper, 1995; Hurme et al, 1997; Keller et al, 1996 and Keller, 1998; Chen et al, 1991). It also represents a size that is slightly larger than the upper end of the size range of a typical cell of *E. coli*, the challenge organism. Theoretically, if the hole is 10 microns and the organism is less than 5 microns it should be able to penetrate.

  One hundred micron holes were also drilled in 200 test trays. One hundred microns is substantially larger than what has been cited as a critical leak size. The 'larger' hole size was chosen to provide an inducement of a difference, with regard to microbial penetration, between the two pressure differentials identified in the study design. Previous research found pouches with 100 micron holes varied in the rate of contamination from 18 percent to 100 percent as the concentration of the challenge suspension increased (Anema et a., 1980). Therefore, at the high level of microbial concentration (10^6 CFU/mL), it was likely that microbial contamination would occur in these samples, providing a positive control for the study.

Table 3, "Physical Variables," from Chapter 2, Literature Review, illustrates the wide range of methods used by researchers to create and quantify holes in the materials or packages for test. In the reviewed literature, pinholes were created by: puncturing the package wall with needles or wires, melting a hole with a Tesla coil, melting a hole with a thermal laser, or by inserting standard pinhole orifices. Channel leakers were created by inserting wires or microtubes in the seal area before sealing the package and then pulling the item from the seal. Most researchers used microscopy of varying types to characterize the size and shape of the hole. In many cases they do not state the type of microscopy used.

Bix et al. (2004) states there are advantages and disadvantages for each different method. The pinholes created mechanically leave defects in the package that are more realistic than pinhole orifices or microtubes. Mechanically created pinholes create an orifice for microbial penetration made of the package material; this is not the case for pinhole orifices and microtubes, which are typically metal or glass. Gilchrist (1989) noted that punched holes in flexible laminate materials resulted in the creation of tears and flaps as well as much variation (from 17-175 microns). The flaps could theoretically close during testing, resulting in the inability of microbes to pass, regardless of the hole size, and thus potentially skew the results (Maunder et al, 1968; Gilchrist et al, 1989; Axelson et al, 1990). Additionally, following testing, Gilchrist indicated that the holes had all decreased in size in varying amounts. Axelson et al. (1990) and Maunder et al. (1968) also reported large variations in the size of pinholes they created manually (from 5 to 100 micron and 33 to 160 micron, respectively).

Extensive work was performed by researchers at Michigan State University on the topic of the creation of defects in packages for research intended to determine penetration threshold (Bix et al, 2004). The research team found a significant effect of measuring technique (Scanning Electron Microscopy, Confocal Microscopy and Optical Microscopy) and that the entry side and exit side of a hole certified to be 50 microns were statistically significantly different in size at a level of α=0.0001. It was hypothesized that the most effective method for determining the hole size was confocal microscopy. Their work was conducted with the same PETG tray that will be used in this research.

For this study, each tray hole size was determined based on flow rate. Flow varies directly with the area of the hole or is a function of the hole diameter squared. This measurement was performed by Lenox Laser, the company that drilled the holes. The flow rate is reported in standard cubic centimeters (sccm) (Reference Results Tables 15, 16, 17, and 18).

- Pressure Differential (2) 0 psi and -3.78 psi

A test group at an elevation of 860 feet above sea level (Lansing, Michigan, elevation) with a pressure of 14.1 psi, and a differential between outside the tray and inside the tray of approximately 0 psi was evaluated. This group was not exposed to an induced pressure differential, only Brownian motion and gravitational settling were the driving forces.

The second test group was exposed to an induced pressure differential simulating what a package would be exposed to when descending in an aircraft or ground shipment from 8,000 ft to an altitude of 860 feet above sea level. Cargo and

commercial aircraft have a pressurized cargo hold, usually maintaining pressures of 8,000 ft. The conditions the second test group was exposed to simulated package exposure to an altitude of 8,000 ft, at which the pressure is 10.32 psi. The pressure inside the tray was 10.32 psi and the pressure outside the tray was 14.1 psi, resulting in a pressure differential of -3.78 psi.

Presumably, the presence of a negative pressure differential will facilitate penetration through defects as well as mimic a common mode of transportation for these types of packages.

It is important to recognize that pressure differentials can also be created through routine handling by the end user, such as squeezing or dropping, of a package.

To determine the necessary volume of air to be withdrawn from the tray in order to induce the pressure differential of -3.78 psi, first the tray volume was measured. The tray volume was determined by measuring the maximum volume of water contained in the tray with a graduated cylinder; Tray Volume (VT)=154 ml.

By withdrawing a volume of air (VS) from the sealed tray with a sterile syringe and needle through a self-sealing septum material, the negative pressure differential the tray experiences is 3.78 psi. This negative pressure differential results in the higher pressure in the cabinet air pushing inward on the test trays at 3.78 psi consistently on every square inch of surface area. This, theoretically, should result in an inward flow of particulates in the air.

The fixed mass of air inside the tray is initially 14.1 psi and occupies a volume of (VT). When air is withdrawn (VS) the same mass of air now occupies a larger

volume (VT + VS) assuming the tray behaves as a rigid system. According to the gas law pV=nRT=constant so 14.1(VT)=10.32(VT+VS), giving VS=56 ml.

As stated previously, this volume determination (VS=56 ml) is based on the assumption that the test tray is a completely rigid system. In reality, this is not the case. When exposed to a negative pressure differential the lidstock visibly deflects inward and it is assumed, while not visible, that the plastic tray also compresses slightly. To correct for this, a simple experiment was conducted, using water because it is incompressible, unlike air. A sealed tray was filled with water using a syringe and needle penetrating the lidstock. Air was allowed to escape and the syringe hole was covered with a non-penetrated piece of septum material, effectively sealing the hole made by the needle. The resultant tray was filled with only water (154 ml), no air, and the lid was completely flat. With a syringe and needle, water was removed from the tray in 1 ml increments. This was repeated until the lid deflection (d) was equivalent (visually detected) to the deflection seen with the trays containing air when 56 ml of air was removed. This deflection is approximately 0.125 inches. Whatever water is in the syringe is the reduction of the internal volume of the tray. The following results chart (Table 10) was generated:

Table 10 – Tray Lidstock Deflection

V (volume of water removed) (ml)	d (deflection of lidstock) (inch)
0	0
1	0.0208
2	0.0417
3	0.0625
4	0.0833
5	0.1041
6	0.125

Based on this information the following correction was made to the formula to account for the tray compressing inward and losing volume V=6 ml. According to the gas law, 14.1(VT)=10.32(VT+VS-V).

VS=154 ml ((14.1psi/10.32psi)-1) + 6=62 ml

The withdrawal of the prescribed air volume (62 ml) was performed using a syringe plunger withdrawal mechanism attached to the fixed racking system designed to support the 4 test trays per group in an inverted orientation (lidstock down); reference Figure 6. The air volume was withdrawn from the trays simultaneously. While it is recognized that the rate of descent from an altitude can vary widely rate was not a factor in this research. The rate for inducing the pressure differential was held constant from run to run. The racking system was designed to facilitate a consistent withdrawal of air from the trays over a one-minute period. Following the one-minute period of air withdrawal, it is also recognized that the rate of 'relaxation' of the trays will vary between the trays with a 10 micron hole and the trays with a 100 micron hole. The rate difference will be considered to be the same as that experienced by actual packages with these size defects experiencing a descent from 8,000 feet. An analysis of the impact of the rate differences is outside the scope of this research.

Constants Details

- Tray and Hole Orientation and Tray Geometry

  A hole was consistently placed in the bottom center of each PETG tray. The trays were consistently placed, relative to the aerosol, in the aerosolizing cabinet, lid-side down.

- Tray Materials

  PETG is a common material for thermoformed medical device trays. Researchers chose to use a non-porous lidstock because, like the choice of an aggressive microbial load, it represents a severe case. The limited porosity of the lid does not allow the system to come to equilibrium with the ambient atmosphere as quickly as with a more porous lid. As a result, equilibration of the system occurs, presumably, through the pinhole alone.

- Microbe; Motility

  Escherichia coli K-12; ATCC Number 29181 was used throughout the experiment. This microbe was selected for this research for 3 reasons:

  1. It is safe and ubiquitous
  2. It is routinely used in MSU laboratory work
  3. The organism is smaller than *Bacillus subtilis* (an organism commonly used in medical device microbial challenge studies)(Reich, 1985; Reich, 1986; Placencia et al 1986; Bankes, 1988; McEldowney et al 1989; ASTM F1608-00). As a result, it is, presumably, a more severe "challenge" of the package system than the device industry's traditional organism, *Bacillus subtilis.*

E-Coli K-12 is a gram-negative, motile, straight-rod that ranges in size from 1.1-1.5μm*2.0-6.0μm (Brooks et al., 2004). In addition to being a more severe challenge, it should serve to accurately represent *Pseudomonas aeruginosa*, a curved rod that ranges in size from 0.5-1.0μm*1.5-5.0μm that is frequently linked to burn infections (Brooks et al, 2004).

The morphology of the *E. coli* used in this experiment was characterized via scanning electron microscopy (SEM), reference Figure 3, and is morphologically consistent with the published literature (Brooks et al, 2004).

- Aerosolization Method

The spray pattern and a racking system were designed such that test samples received full coverage of microbes during test cycles.

The American Conference of Governmental Industrial Hygienists define bioaerosol as an airborne dispersion of particles containing whole or parts of biological entities, such as bacteria, viruses, dust mites, fungal hyphae, or fungal spores.

The behavior of bioaerosols is governed by the principles of gravitation, electromagnetism, turbulence, and diffusion. Since bioaerosol particles are larger than 1 micron, diffusion due to gravitational settling is the dominant mechanism. Brownian motion, the irregular movement of microparticles, is not a major factor for the movement of bioaerosols. Thermal gradients can also induce aerosol movements; this also will be considered negligible as there is not a thermal gradient in the aerosol chamber. It is likely that shortly after exiting the spray nozzle, the particles will be in laminar flow, carried along the airstream with the air molecules. The heavier

microparticles will break free and settle gravitationally (Penn State Department of Physics, 2003).

- Microbial Exposure Time

The aerosolization duration for this research was determined through experimentation with the process intended to correlate the volume of solution introduced into the cabinet, to the dosage delivered for a given starting concentration of microbes. The total chamber exposure time of 30 minutes was selected for the following reasons: Chamber exposure time of 30 minutes per package and a test organism concentration of 10^6 was chosen because Keller's work (Keller et al, 1996) showed significant penetration at this concentration when working with *Pseudomonas fragi* TM 849. Work performed by Anema and Schram (1980), found that increasing exposure time for a given hole size and given microbe concentration from 1 minute to 30 minutes resulted in an increase of contamination from 15 percent to 58 percent.

ASTM 4169-05 Standard Practice for Performance Testing of Shipping Containers and Systems Schedule I 'Low Pressure (High Altitude) Hazard Testing' prescribes a 1-hour duration. While there is no approved standard at this time for whole package microbial testing, laboratories are exposing packages to an aerosol of organisms for a few minutes then leaving the packages in the chamber with the settled organisms for 1 hour. The descent of the aircraft typically ranges from 1,000 to 1,500 ft/min. As such, it would take the plane approximately 8 minutes to complete its descent. If a truck is descending from the mountains at 60 mph, depending on the grade of the road could take significantly longer than eight minutes. During a typical aircraft landing, recording barometers have shown that the pressure in the cargo hold

will increase from 12.2 psi to 14.7 psi in approximately 30 minutes (Hackett et al, 2000).

When the test trays are removed from the chamber after 30 minutes and transferred to the incubation chamber, there was no wiping of the tray or disinfection. In particular, the disinfection step was a source of controversy regarding its possible contribution to the generation of false positives and false negatives in the traditional microbial challenge method (Reference Appendix I, New Method Development). So the TOTAL exposure time for the trays was reported as the time from initial exposure and included the entire incubation period.

The test conditions produced conditions far in excess of what might realistically be found in product use. However, this employs a conservative approach intended to create harsh conditions in order to determine the impact of pressure differential on microbial ingress.

- Temperature and Humidity (T, RH)

  Testing: (25°C, 50% RH)

  The testing was performed under controlled laboratory conditions (25°C ± 2°C). Due to the significant effect that temperature has on pressure differential, it is important that the temperature not vary significantly. While the temperature was not monitored during experimentation and laboratories do vary, no significant temperature excursions were noted. It is recognized that the %RH of the cabinet air likely increased significantly during aerosolization. Placencia et al (1986) noted a 24 percent RH increase in the aerosol test cabinet used for their research in the first 1.5 minutes of aerosolization. The research team noted that the '%RH

returned to ambient conditions 5 minutes after the chamber was cleared' (Placencia et al, 1986).

Incubation: (37°C, 50% RH)

The incubation conditions are optimum for *E. coli* growth and the environmental chamber will minimize variation in conditions.

Test Groups

Four trays were tested per trial. *Trial One* consisted of a run with 4 trays with 100 micron holes at a microbial load of 0 with 2 trays each at 0 psi and –3.78psi. The second run consisted of 4 trays with 100 micron holes at a microbial load of 10^6 with 2 trays each at 0 psi and –3.78 psi. The tray positions with respect to pressure differential were rotated to provide positional balance. Each run was repeated 3 times giving a total of 8 runs, 32 trays.

Trial Two consisted of a run with four combinations of hole size (no hole and 10 micron) and pressure differential (0 and –3.78 psi) in the cabinet with a microbial load of 0. The second run consisted of four combinations of hole size (no hole and 10 micron) and pressure differential (0 and –3.78 psi) in the cabinet with a microbial load of 10^6. The tray positions with respect to pressure differential were rotated to provide positional balance. Each run was repeated 3 times giving a total of 8 runs, 32 trays. This gives 4 replicates of each of 8 treatments ignoring possible run effects. Total trays for Trial One and Trial Two were 64 trays.

Statistical Design

There are three factors which were varied in the experiment. The factor *microbial load* was tested at two levels 0 and 10^6, the factor *hole size* at no hole, 10, and

100 microns, and the factor *pressure differential* at two levels, 0 and -3.78 psi. The aerosol cabinet allows runs in blocks of size 4. As part of the analysis, results were examined to see if it was plausible to ignore block effects.

The experiment was broken into trials (stages) that proceeded in a sequential manner. This approach allows for adaptation based on findings from earlier stages. As decribed earlier, Trial One kept *hole size* fixed at 100 microns. The intent was to develop evidence that the microbes were viable and able as expected to easily penetrate the barriers through a 100 micron hole. The factors concentration and pressure differential were varied allowing for estimation of effects for these variables. Based on the results a decision was made following Trial One to eliminate the 100 micron hole size from subsequent trials.

Trial Two implemented four replications of a complete 2^3 factorial design with *microbial load* at levels 0 and 10^6, *hole size* at levels no hole and 10 microns, and *pressure differential* at levels 0 and -3.78 psi. A decision stemming from Trial Two was to eliminate the 0 microbial load from subsequent experiments. In this case, the model becomes a simple two by two factorial run in blocks of size 4 (cabinet capacity).

The response variables analyzed included whether the trays showed any colony forming units (an attribute variable Yes or No) and the colony count at 72 hours.

The decision on the number of additional runs needed to detect meaningful differences in the treatments was made following the completion of Trials One and Two. To increase the power of the results from the first trials, two identical replicate trials (Trials Three and Four) were conducted. These trials consisted of 36 trays each. Four of the trays were exposed to no microbial load, a control group. The remaining 32 trays

contained a 10 micron hole and were exposed to a microbial concentration of 10^6 CFU/mL

- Data Analysis

  Frequency counts for the presence of colony forming units were compared across conditions using the Fisher Exact Test to test the effects of pressure differential and hole size on microbial penetration. Effects of tray position and hole size variation from nominal were tested using counts of colony forming units with t-tests and F-tests. All effects were considered at the 0.05 level of significance.

CHAPTER 5

RESULTS

The trials and runs that make up the primary research (Experiment Two) are described below using tree diagrams (Tables 11 through 14). Following these tables, tables of colony counts (CFUs) following 72 hours of test tray incubation are given as the response for the various factor level combinations that were tested. Run numbers and tray positions within the runs are also given.

Table 11 – Trial One

Microbial Load(cells)	Hole Size(micron)	Pressure Differential(psi)	#TRAYS
Test Runs 1A, 1B, 1C, 1D			
0 →	100 →	2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
			16
Test Runs 2A, 2B, 2C, 2D			
10^6 →	100 →	2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
			16

Table 12 – Trial Two

Microbial Load(cells)	Hole Size(micron)	Pressure Differential(psi)	#TRAYS
Test Runs 3A, 3B, 3C, 3D			
0	0	2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
	10	2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
			16
Test Groups 4A, 4B, 4C, 4D			
10^6	0	2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
	10	2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
			16

Table 13 – Trial Three (Replicate One)

Microbial Load(cells)	Hole Size(micron)	Pressure Differential(psi)	#TRAYS
Test Run 5A			
0	10	2(0) 2(-3.78)	4
			4
Test Runs 5B through 5I			
10^6	10	2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
			32

Table 14 – Trial Four (Replicate Two)

Microbial Load(cells)	Hole Size(micron)	Pressure Differential(psi)	#TRAYS
Test Run 6A			
0	10	2(0) 2(-3.78)	4
			4
Test Runs 6B through 6I			
10^6	10	2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
		2(0) 2(-3.78)	4
			32

Table 15 – Trial One Results

Test Run #	Tray Number/ Position	Pressure Differential (psi)	Aerosolized Microbe Concentration (cells/ml)	Flow Rate (sccm)	Hole Diameter (micron)	CFU (Total Count)
1A	1	0	0	175.547	100.154	0
1A	2	0	0	163.328	96.605	0
1A	3	-3.78	0	172.794	99.365	0
1A	4	-3.78	0	165.632	97.284	0
1B	1	-3.78	0	171.290	98.932	0
1B	2	0	0	168.761	98.199	0
1B	3	0	0	180.278	101.494	0
1B	4	-3.78	0	177.090	100.593	0
1C	1	-3.78	0	153.391	93.620	0
1C	2	-3.78	0	160.792	95.852	0
1C	3	0	0	169.735	98.510	0
1C	4	0	0	179.604	101.304	0
1D	1	0	0	175.718	100.202	0
1D	2	-3.78	0	170.977	98.841	0
1D	3	-3.78	0	176.004	100.284	0
1D	4	0	0	165.458	97.233	0

Test Run #	Tray Number/ Position	Pressure Differential (psi)	Aerosolized Microbe Concentration (cells/ml)	Flow Rate (sccm)	Hole Diameter (micron)	CFU (Total Count)
2A	1	0	10^6	155.083	94.135	2
2A	2	0	10^6	161.311	96.034	0
2A	3	-3.78	10^6	164.869	97.060	68
2A	4	-3.78	10^6	166.778	97.620	121
2B	1	-3.78	10^6	166.536	97.549	193
2B	2	0	10^6	160.455	95.779	0
2B	3	0	10^6	170.197	98.616	0
2B	4	-3.78	10^6	168.076	97.999	180
2C	1	-3.78	10^6	166.809	97.629	76
2C	2	-3.78	10^6	157.798	94.983	148
2C	3	0	10^6	173.677	99.619	0
2C	4	0	10^6	183.373	102.362	0
2D	1	0	10^6	169.156	98.313	0
2D	2	-3.78	10^6	181.738	101.904	92
2D	3	-3.78	10^6	176.079	100.305	136
2D	4	0	10^6	172.251	99.209	0

Table 16 – Trial Two Results

Test Run #	Tray Number/ Position	Pressure Differential (psi)	Aerosolized Microbe Concentration (cells/ml)	Flow Rate (sccm)	Hole Diameter (micron)	CFU (Total Count)
3A	1	0	0	0	0	0
3A	2	0	0	0	0	0
3A	3	-3.78	0	1.831	10.406	0
3A	4	-3.78	0	1.726	10.102	0
3B	1	-3.78	0	0	0	0
3B	2	0	0	0	0	0
3B	3	0	0	1.964	10.776	0
3B	4	-3.78	0	1.916	10.644	0
3C	1	-3.78	0	0	0	0
3C	2	-3.78	0	0	0	0
3C	3	0	0	1.697	10.018	0
3C	4	0	0	1.89	10.572	0
3D	1	0	0	0	0	0
3D	2	-3.78	0	0	0	0
3D	3	-3.78	0	1.710	10.057	0
3D	4	0	0	1.842	10.438	0

Test Run #	Tray Number/ Position	Pressure Differential (psi)	Aerosolized Microbe Concentration (cells/ml)	Flow Rate (sccm)	Hole Diameter (micron)	CFU (Total Count)
4A	1	0	10^6	0	0	0
4A	2	0	10^6	0	0	0
4A	3	-3.78	10^6	1.673	9.946	3
4A	4	-3.78	10^6	2.579	12.350	0
4B	1	-3.78	10^6	0	0	0
4B	2	0	10^6	0	0	0
4B	3	0	10^6	1.430	9.197	0
4B	4	-3.78	10^6	1.892	10.577	0
4C	1	-3.78	10^6	0	0	0
4C	2	-3.78	10^6	0	0	0
4C	3	0	10^6	1.636	9.836	0
4C	4	0	10^6	2.549	12.277	0
4D	1	0	10^6	0	0	0
4D	2	-3.78	10^6	0	0	0
4D	3	-3.78	10^6	1.519	9.478	0
4D	4	0	10^6	1.959	10.762	0

Table 17 – Trial Three Results

Test Run #	Tray Number/ Position	Pressure Differential (psi)	Aerosolized Microbe Concentration (cells/ml)	Flow Rate (sccm)	Hole Diameter (micron)	CFU (Total Count)
5A	1	0	0	1.862	10.493	0
5A	2	-3.78	0	1.165	8.301	0
5A	3	0	0	1.935	10.696	0
5A	4	-3.78	0	1.439	9.226	0
5B	1	0	10^6	1.636	9.837	0
5B	2	0	10^6	1.820	10.376	0
5B	3	-3.78	10^6	2.090	11.117	0
5B	4	-3.78	10^6	1.716	10.075	0
5C	1	-3.78	10^6	1.787	10.279	2
5C	2	0	10^6	1.413	9.141	0
5C	3	0	10^6	1.960	10.765	0
5C	4	-3.78	10^6	1.402	9.107	0
5D	1	-3.78	10^6	1.866	10.503	0
5D	2	-3.78	10^6	1.762	10.207	0
5D	3	0	10^6	2.277	11.604	0
5D	4	0	10^6	1.589	9.694	0
5E	1	0	10^6	1.786	10.277	0
5E	2	-3.78	10^6	1.837	10.424	0
5E	3	-3.78	10^6	1.396	9.085	0
5E	4	0	10^6	1.504	9.432	0
5F	1	0	10^6	2.102	11.150	0
5F	2	0	10^6	1.707	10.047	0
5F	3	-3.78	10^6	2.270	11.587	0
5F	4	-3.78	10^6	1.664	9.921	2
5G	1	-3.78	10^6	1.749	10.170	0
5G	2	0	10^6	1.804	10.330	0
5G	3	0	10^6	1.617	9.779	0
5G	4	-3.78	10^6	1.919	10.653	0
5H	1	-3.78	10^6	1.541	9.545	0
5H	2	-3.78	10^6	1.590	9.697	3
5H	3	0	10^6	2.082	11.096	0
5H	4	0	10^6	1.424	9.177	0
5I	1	0	10^6	1.824	10.387	0
5I	2	-3.78	10^6	1.961	10.770	0
5I	3	-3.78	10^6	1.517	9.473	0
5I	4	0	10^6	2.215	11.446	0

Table 18 – Trial Four Results

Test Run #	Tray Number/ Position	Pressure Differential (psi)	Aerosolized Microbe Concentration (cells/ml)	Flow Rate (sccm)	Hole Diameter (micron)	CFU (Total Count)
6A	1	0	0	1.536	10.493	0
6A	2	-3.78	0	2.144	8.301	0
6A	3	0	0	2.393	10.696	0
6A	4	-3.78	0	1.361	9.226	0
6B	1	0	10^6	1.610	9.837	0
6B	2	0	10^6	1.271	10.376	0
6B	3	-3.78	10^6	1.344	11.117	1
6B	4	-3.78	10^6	1.679	10.075	0
6C	1	-3.78	10^6	1.822	10.279	3
6C	2	0	10^6	1.370	9.141	0
6C	3	0	10^6	1.628	10.765	0
6C	4	-3.78	10^6	1.463	9.107	0
6D	1	-3.78	10^6	1.784	10.503	0
6D	2	-3.78	10^6	1.874	10.207	0
6D	3	0	10^6	1.846	11.604	0
6D	4	0	10^6	1.803	9.694	0
6E	1	0	10^6	1.454	10.277	0
6E	2	-3.78	10^6	1.466	10.424	0
6E	3	-3.78	10^6	1.415	9.085	0
6E	4	0	10^6	2.701	9.432	0
6F	1	0	10^6	1.601	11.150	0
6F	2	0	10^6	1.376	10.047	0
6F	3	-3.78	10^6	1.462	11.587	2
6F	4	-3.78	10^6	2.005	9.921	0
6G	1	-3.78	10^6	1.445	10.170	0
6G	2	0	10^6	1.545	10.330	0
6G	3	0	10^6	1.831	9.779	0
6G	4	-3.78	10^6	1.793	10.653	0
6H	1	-3.78	10^6	1.460	9.545	0
6H	2	-3.78	10^6	1.925	9.697	0
6H	3	0	10^6	1.454	11.096	0
6H	4	0	10^6	1.136	9.177	0
6I	1	0	10^6	1.726	10.387	0
6I	2	-3.78	10^6	1.739	10.770	2
6I	3	-3.78	10^6	1.368	9.473	0
6I	4	0	10^6	2.004	11.446	0

Petri dishes containing sterile media were placed on the floor of the cabinet for each run as an indicator of organism viability, microbe dosage delivered and an absence of foreign organism in the system. Table 19 illustrates that the organisms were viable, the dose delivered was consistent from run to run and that no foreign organisms were present in the system.

Table 19 - Petri Dish Growth Quantification Results

Trial Number/Run	Microbial Load CFU/mL	Colonies (CFU) per sqin
1A	None	0
1B	None	0
1C	None	0
1D	None	0
2A	10^6	102
2B	10^6	121
2C	10^6	113
2D	10^6	97
3A	None	0
3B	None	0
3C	None	0
3D	None	0
4A	10^6	117
4B	10^6	139
4C	10^6	124
4D	10^6	112
5A	None	0
5B	10^6	113
5C	10^6	134
5D	10^6	98
5E	10^6	125
5F	10^6	116
5G	10^6	99
5H	10^6	101
5I	10^6	127
6A	None	0
6B	10^6	121
6C	10^6	114
6D	10^6	92
6E	10^6	99
6F	10^6	138
6G	10^6	106
6H	10^6	111
6I	10^6	98

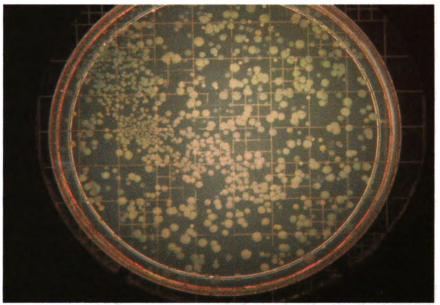


Figure 19 - Petri Dish from a Trial Exhibiting Growth

Results Summary

All reported results are following 72 hours of incubation.

Trial One

All test trays (8/8) with 100 micron holes, exposed to a microbial concentration of 10^6, experiencing a pressure differential, exhibited growth, while only one (1/8) 100 micron test tray not experiencing a pressure differential exhibited growth. The number of colonies observed in this test tray (2 CFU) was much less than those experiencing a pressure differential (Average=127 CFU). No trays (0/16) exposed to 0 microbial concentration exhibited growth. Petri dishes for each run with microbial exposure were consistent with levels noted in other runs and in growth curve development (Procedure A).

Trial Two

One test (1/4) with 10 micron holes, exposed to a microbial concentration of 10^6, experiencing a pressure differential, exhibited growth, while no (0/4) 10 micron test tray not experiencing a pressure differential exhibited growth. No trays (0/16) without a test hole exhibited growth under any conditions. No trays (0/16) exposed to 0 microbial concentration exhibited growth. Petri dishes for each run with microbial exposure were consistent with levels noted in other runs and in growth curve development (Procedure A).

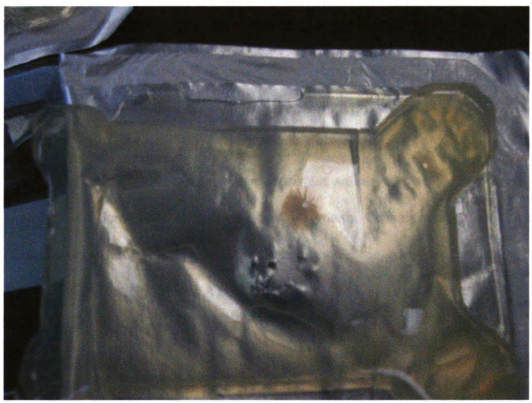


Figure 20 - Test Tray Exhibiting Microbial Growth; Trial Three

Trial Three

Three test trays (3/16) with 10 micron holes (Figure 20), exposed to a microbial concentration of 10^6, experiencing a pressure differential exhibited growth (average=2.3 CFU), while no (0/16) 10 micron test trays not experiencing a pressure differential exhibited growth. Number of CFUs was very consistent among test trays, as seen in the data presented. No trays (0/4) exposed to 0 microbial concentration exhibited growth. Petri dishes for each run with microbial exposure were consistent with levels noted in other runs and in growth curve development (Procedure A).

Trial Four

Four test trays (4/16) with 10 micron holes, exposed to a microbial concentration of 10^6, experiencing a pressure differential exhibited growth (average 2 CFU), while no (0/16) 10 micron test trays not experiencing a pressure differential exhibited growth. Number of CFUs was very consistent among test trays. No trays (0/4) exposed to 0 microbial concentration exhibited growth. Petri dishes for each run with microbial exposure were consistent with levels noted in other runs and in growth curve development (Procedure A).

Enterotube™ II Results

All identified CFUs from Trials Two, Three and Four were confirmed as *Escherichia coli* with no atypical tests; Identification Code 24460 from the Enterotube™ II Interpretation Guide B-D Catalog Number 24330; Revised August 1993. Identification was performed per Procedure K with the B-D Enterotube™ II system, reference Figure 21.

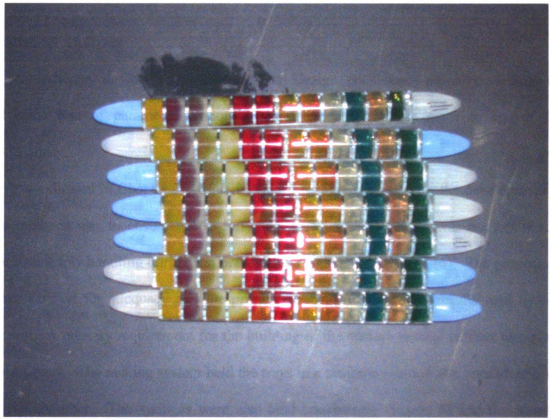


Figure 21 - Enterotube™ II Results

Statistical Analyses

As indicated in Chapter 4 – Experimental Design, consistency among conditions for all trials was of paramount importance and every effort was made to ensure this consistency.

As indicated in Chapter 3 – Materials and Methods, the basis for the development of the methods was to ensure run to run consistency. A custom jig was built to ensure consistent tray handling during test preparation (Figures 14, 15, and 16). The procedures were verified for adequacy and the primary research was run and results read by one person. A primary requirement for the building of the custom aerosol cabinet was again consistency. The racking system held the trays in a uniform position at a consistent level in the chamber. The syringes were also held consistently (Figure 6) and the plunger retraction was performed simultaneously at a consistent rate (1 minute) with a bracket which fit over the plungers while the cabinet was sealed. Handling was facilitated during this process with gloves integrated into the cabinet wall (Figure 7).

The results were examined and analyzed to look for effects due to run and to tray position. These examinations and analyses showed no evidence of these effects. For example, note that across all eight trays that showed positive counts with a nominal hole size of 10 microns (microbial concentration of 10^6 CFU/mL and pressure differential of -3.78 psi), the tray positions are balanced, specifically being Tray 1 (n=2), Tray 2 (n=2), Tray 3 (n=3) and Tray 4 (n=1). In Trial One, Test Run 2, with a nominal hole size of 100 microns (microbial concentration of 10^6 CFU/mL and pressure differential of -3.78 psi), all eight trays were positive for microbial growth necessarily dividing equally among the tray positions in the balanced design (Tables 11, 12, 13, and 14). The mean

counts for the trays in the four positions are not statistically different (p-value = 0.84 based on an F-test).

As indicated in the Results Tables 15, 16, 17, and 18 in this chapter, the measured hole diameters varied about the nominal values. This leads to the question of whether these differences relate to the CFU counts. To address this question, two analyses were performed. For the eight positives for the 100 micron group (microbial concentration of 10^6 CFU/mL and pressure differential of -3.78 psi), the correlation of measured diameter with count is (correlation coefficient = -0.19, p-value = 0.65 based on a t-test). For the eight positives in the 10 micron group (microbial concentration of 10^6 CFU/mL and pressure differential of -3.78 psi), the CFU counts were 1 (n=1), 2 (n=4) and 3 (n=3). The mean hole diameters for the trays in these groups are not statistically different (p-value=0.25 based on an F-test). These statistical tests, admittedly of little power because of sample size, retain the hypothesis that the observed counts are not related to the deviation from nominal hole diameter.

Trial One

Test Group 1 (100 micron, concentration of microbes=0)

Result: 0 positive in 8 trays with pressure differential of 0 psi

Result: 0 positive in 8 trays with pressure differential of -3.78 psi

Test Group 2 (100 micron, concentration of microbes=10^6)

Result: 1 positive in 8 trays with pressure differential of 0 psi

Result: 8 positive in 8 trays with pressure differential of -3.78 psi

The large difference in magnitudes of the CFUs should be noted: (average 127 CFU per tray for pressure differential of -3.78 psi) versus (2 CFU for tray with

0 pressure differential). In total, very strong evidence that pressure differential has a large effect when a 100 micron pinhole is present and microbial concentrations of 10^6 are tested. In addition, the effect of pressure differential at 100 micron and concentration=10^6 was assessed with a one-sided Fisher Exact Test. The comparison of 8 out of 8 versus 1 out of 8 has P-value = 0.0007, demonstrating a statistically significant effect.

Trial Two

Test Group 3 (10 micron, concentration of microbes=0)

Result: 0 positive in 8 trays with pressure differential of 0 psi

Result: 0 positive in 8 trays with pressure differential of -3.78 psi

Results support the control exhibited in the results of Test Group 1.

Test Group 4 (10 and 0 micron , concentration of microbes=10^6)

Result: 0 positive in 8 trays (0 and 10 micron) with pressure differential of 0 psi

Result: 1 positive in 4 trays (10 micron) and 0 positive in 4 trays (0 micron) with pressure differential of -3.78 psi.

For the 10 micron trays, the Fisher Exact assessment (one-sided) comparing 1 positive tray out of 4 trays at -3.78 psi versus 0 positive trays out of 4 trays at 0 psi gives P-value = 0.5, not statistically significant.

Trial Three -Replicate 1 (10 micron, concentration of microbes=10^6) Replicates Test Group 4 experiment with sample sizes of 16 and 16

Result: 0 positive in 16 trays with pressure differential of 0 psi

Result: 3 positives in 16 trays with pressure differential of -3.78 psi

Fisher Exact assessment (one-sided) of P-value is 0.1129; not statistically significant in itself.

Trial Four -Replicate 2 (10 micron, concentration of microbes=10^6) Another replicate of Test Group 4 experiment with sample sizes of 16 and 16

Result: 0 positive in 16 trays with pressure differential of 0 psi

Result: 4 positives in 16 trays with pressure differential of -3.78 psi

Fisher Exact assessment (one-sided) of P-value is 0.0506; not statistically significant in itself.

Controlling for possible experimental effects for the three separate experiments (Test Group 4, Replicate 1 and Replicate 2), the assessment (one-sided) of P-value is (0.5)(0.1129)(0.0506)=**0.0029** for comparing pressure differential of -3.78 psi versus 0 psi at 10 micron and concentration of microbes=10^6; **statistically significant.** If one ignores possible experimental effects for the three experiments and simply aggregates the results, the Fisher Exact one-sided assessment of 8 out of 36 versus 0 out of 36 gives P-value = 0.0025.

The effect of hole size 100 microns versus 10 microns at pressure differential -3.78 psi and concentration=10^6 was assessed using data from Test Groups 2 and 4 and Replicates 1 and 2. The one-sided Fisher Exact comparison of 8 out of 8 versus 8 out of 36 gives P-value < 0.0001, demonstrating a statistically significant effect. The difference 1 out of 8 versus 0 out of 36 at pressure differential 0 psi is not statistically significant.

Conclusion

Table 20
Aggregated Results of Positive Responses for n = 136 Trays

	Microbe Load			
	0		10^6	
Pressure Diff	**Δ = -3.78 psi**	**Δ = 0 psi**	**Δ = -3.78 psi**	**Δ = 0 psi**
Hole Size				
0 μ	0 out of 4	0 out of 4	0 out of 4	0 out of 4
10 μ	0 out of 8	0 out of 8	8 out of 36[a,c]	0 out of 36[a]
100 μ	0 out of 8	0 out of 8	8 out of 8[b,c]	1 out of 8[b]

[a] Data from Test Group 4 and Reps 1 and 2. Statistically significant difference.
[b] Results of Test Group 2. Statistically significant difference.
[c] Data from Test Groups 2 and 4, Replicates 1 and 2. Statistically significant difference.

The presence of a pressure differential has a statistically significant effect on the ingress of the test organism in a sterile, rigid medical device tray. The effect of hole size (100 micron versus 10 micron) at a pressure differential of -3.78 psi is statistically significant.

CHAPTER 6

DISCUSSION

This section is a discussion of the results obtained from the primary research conducted and detailed in this dissertation. The goal of this first section is to explain the observed number of CFU observed in the test trays. It is assumed that the number of CFU is the result of the induced pressure differential pulling a quantity of cabinet air containing microbes into the test tray. This can be check as follows:

- Two spray canisters deliver a total of 50 ml of liquid with a concentration of 10^6 CFU/mL in 15 seconds. So $50 \times 10^6 = 5 \times 10^7$ CFU enter the chamber.
- Assuming the CFU spread out evenly inside the chamber (Volume = 28.5" x 20" x 36" = 20,520 in^3), they are suspended in air at a concentration of :

$$\frac{5 \times 10^7 \text{ CFU}}{20{,}520 \text{ in}^3} = 2437 \text{ CFU per in}^3 \text{ of air}$$

- Once the vacuum is applied (via the pull of the syringe), air (containing CFUs from the chamber) enters the hole, carrying CFUs with it, and fills the tray. Air continues to flow in until the pressure inside the tray matches the pressure outside (equilibrium is reached).
- The volume of air that must enter the tray to equilibrate pressures:

  Starts at pressure $P_a - \Delta p$ where P_a = 14.1 psi (ambient-Lansing, MI) and Δp = 3.78 psi

  The number of moles of air in the tray at start ($n_{initial}$)

$$= n_{initial} = \frac{(P_a - \Delta p)\, V;}{RT}$$

where V = Tray Volume (same as VT)
R = Universal Gas Constant
T = Temperature (absolute)

According to the gas law when pressures equilibrate,

$$n_{final} = \frac{P_a V}{RT}$$

Therefore, the number of moles of cabinet air that must enter is:

$$N_{final} - n_{initial} = \frac{\Delta p\, V}{RT}$$

From the gas law, this is equivalent to a volume of cabinet air equal to incoming air volume =

$$\frac{(\Delta p\, V)}{(RT)} \frac{RT}{P_a} = V \frac{\Delta p}{P_a} = 154(3.78/14.1) = 41.3 \text{ mL}$$

This is equivalent to 2.5 in^3 of cabinet air containing

2.5 in^3 x (2437 CFU/in^3) = 6092 CFU.

In theory, this is true regardless of hole size. The smaller the hole, the CFUs flow into the tray slower, the equilibrium time is longer, but the net CFUs flowing in is the same.

Why weren't this many CFUs observed in this research? Some possible reasons:

1. The test time of 30 minutes was not long enough to reach equilibrium
2. There was injury to the micro-organisms
3. The mist containing the concentration of microbes settled out of the air before the equilibrium time

Exploring these theories:

1. The Bernoulli Equation (Potter and Foss, 1975):

$$\frac{V_1^2}{2g} + \frac{P_1}{\gamma} = \frac{V_2^2}{2g} + \frac{P_2}{\gamma}$$

where g = gravity = 32.2 ft/sec^2

γ = air density = $\frac{1 \text{ lb}}{13.3 \text{ ft}^3}$ @ 72°F

relates the flow speed V to the pressure differential, as air flows from State 1 to State 2.

State 1: Starts somewhere within air mass inside chamber:

$$P_1 = p_a \quad \text{and} \quad V_1 = 0$$

State 2: Just after it passes through the hole and enters the tray, it sees pressure

$$P_2 = P_a - \Delta p \text{ and has speed of } V_2 = V.$$

Then the Bernoulli equation becomes:

$$\frac{0^2}{2g} + \frac{P_a}{\gamma} = \frac{V^2}{2g} + \frac{P_a - \Delta p}{\gamma}$$

Solving for the air speed as if flows through the hole gives

$$V = [2g/\gamma \cdot \Delta p]^{1/2}$$

where g = gravity = 32.2 ft/sec^2

γ = air density = $\frac{1 \text{ lb}}{13.3 \text{ ft}^3}$ @ 72°F

and Δp = 3.78 psi = 544.3 lb/ft^2 So it passes through the tray hole with a speed of:

$$V = [(2 \times 32.2/1 \div 13.3)(544.3)]^{1/2} = 680 \text{ ft/sec}$$

The flow rate (volume per unit time) through the hole is AV, where A = hole area and V = speed (680 ft/sec). For the 100 micron hole (nominal), the radius is 1.64×10^{-4} ft, so the hole area = $\pi r^2 = 8.45 \times 10^{-8}$ ft^2. So the flow rate initially is:

$AV = 8.45 \times 10^{-8}$ (680 ft/sec) = 5.75×10^{-5} ft^3/sec = 1.62 ml/sec.

As air enters the tray, the inside pressure rises, Δp drops, the speed drops, and the flow rate drops. The flow rate eventually becomes zero, so the average flow rate is about:

$\frac{1.62+0}{2} = 0.81$ ml/sec for the 100 micron hole

Since the tray with the 100 micron hole needs an air volume of 41.3 ml to flow in to equilibrate pressures, this should take only

$\frac{41.3 \text{ ml}}{0.81 \text{ ml/sec}} = 51$ seconds

These calculations assume no air friction, which is not the case, so this estimate represents a lower limit.

For the 10 micron hole (nominal), the only thing that changes is A, the hole diameter, which is 100 times smaller, so it should take 100 x 51 = 5,100 sec = 1.42 hours. Therefore, insufficient test time could explain the 10 micron results, but does not entirely explain the 100 micron results.

2. Theoretically, the 100 micron hole should have allowed 6092 CFU into the tray. There was enough time for this to occur, as discussed previously. It is possible that microbe injury was a contributing factor for the reduced amounts

of CFUs actually transferred. The work performed to verify the cabinet functionality (Chapter 3, Procedure A) indicated significant injury to the organisms during the aerosolization process. The growth rate was 101 CFU/sqin for the dose delivered in this research. This yields a significant, approximate death rate of [(87,720-101)/87,720] x 100 = 99% (Reference Section 3, below). Additional microbe injury could be occurring to the organisms which survive the aerosolization process as they enter the tray hole. Examining the hole in detail, the ratio of hole diameter to length is:

$$\frac{100 \text{ micron}}{20 \text{ mil}} = \frac{4 \text{ mil}}{20 \text{ mil}} = 1/5$$

For the 10 micron hole, the ratio is 1/50.

The longer the hole compared to its diameter, the greater the opportunity for impacts between the penetrating microbe and the sides of the hole, which would likely result in devastating consequences for the micro-organism, given the speed of 680 ft/sec.

3. To make a liberal estimate regarding the rate of the mist dropping out of the air in the chamber, the assumption is made that the mist settles and falls vertically down, collecting only on horizontal surfaces inside the cabinet. The concentration of CFUs on these horizontal surfaces is 5 x 10^7 CFUs injected in cabinet divided by the total horizontal area, which is 28.5" x 20", so:

$$\text{Concentration} = \frac{5 \times 10^7 \text{ CFU}}{28.5" \text{ X } 20"} = 87{,}720 \text{ CFU/in}^2$$

The 100 micron, hole occupies an area of 8.45×10^{-8} ft^2 = 1.22×10^{-5} in^2, so the number of CFU's that land in the hole should be:

$$87{,}720 \text{ CFU/in}^2 \times 1.22 \times 10^{-5} = 1.06 \text{ or just 1 CFU}$$

The 10 micron hole is 100 times less, or 0.01 CFU should land on the hole. These estimates are low compared to the data collected. Considering the death rate discussed in Part 2 which occurs during aerosolization, the estimate for the 100 micron hole becomes 0.001 CFU and for the 10 micron hole 0.00001 CFU that should land on the holes. The data does in fact show that the 10 micron holes had about 100 times fewer CFU than the 100 micron holes, which supports the idea that settling accounts for the observed results, but the CFU counts observed are far greater than these estimates calculated above. Given such low probabilities, it is significant that a 100 micron test tray was penetrated without the presence of a pressure differential. It appears that the resultant CFU counts in the test trays are a combination of microbes settling out of the mist onto the test trays and microbes in the mist being drawn into the trays as a result of the pressure differential driving force.

In addition to the considerations surrounding the driving force and microbial penetration, another factor that must be considered is surface tension. Surface tension is the result of cohesive forces between liquid molecules. Molecules at the surface do not have other like molecules on all sides of them and, consequently, they cohere more strongly to those directly associated with them on the surface. The surface molecules exist in a state of tension. In this state of tension, they resist deformation.

Keller's (1998) research illustrated that surface tension was a critical food product parameter in determining the threshold leak size and pressure (Keller et al, 1998).

The surface tension of water is 72 dynes/cm at 25°C. This means that it would take a force of 72 dynes to break a surface of film of water 1 cm long. Potassium Phosphate, Monobasic, Crystal (KH_2PO_4) diluted with water is commonly referred to as Butterfield's Buffer Solution. This was the carrier solution for the aerosolized *E. coli*. Butterfield's Solution has a surface tension of 65 dynes/cm at 25°C, slightly less than water, making it a slightly better wetting agent than water at 25°C. The better the wetting agent, the more likely the solution is to enter the pinholes rather than bridging them with surface tension (http://hyperphysics.phy-astr.gsu.edu, 2005). Therefore, a threshold pressure must be exceeded to allow the aerosol droplets of microbes contained in Butterfield's Solution to ingress into the PETG tray.

In the Introduction of this dissertation, the influence of pressure differential magnitude on microbial ingress was briefly discussed. The discussion above regarding microbial injury due to the extremely high calculated flow rates which was theorized as a contributing factor to the low CFU counts does seem to support the concept of 'impaction'. This theory states that a 'moderate' flow rate maximizes microbial penetration. On the low side, diffusion is theorized to be the predominant mechanism of penetration. On the high side, impaction predominates, the microbes are removed because they are 'smashed' into porous fibers, or in this research, where a non-porous system was examined, may experience catastrophic injury while being pulled into the hole. According to this theory, maximum penetration will occur when neither impaction nor diffusion is the predominant mechanism. This research suggests the concept that

high flow rates may negatively impact penetration in nonporous systems as well as porous systems. However, because this research did not investigate an intermediary pressure differential, and was not intended to specifically address this question, further research is recommended to confirm and quantify this phenomenon.

With regard to one of the goals of this research, to enhance patient safety, a brief discussion of the concept of microbial infective dose and zero tolerance is salient. *E-Coli K-12*, a gram-negative, motile, straight-rod that ranges in size from 1.1-1.5 μm*2.0-6.0 μm (Brooks et al., 2004) was the selected test organism. As discussed earlier, one of the reasons it was selected for this research was that it would serve as a model of a microbe frequently linked to burn infections, *Pseudomonas aeruginosa*, a curved rod that ranges in size from 0.5-1.0μm*1.5-5.0μm (Brooks et al., 2004). The penetration of *E. coli* models penetration of relevant pathogens.

Infective dose is the number of a particular microorganism required to cause disease in a susceptible host (Brooks et al., 2004). The concept of zero tolerance is commonly applied to food born disease agents. For some disease agents, zero organisms are acceptable in some foods. In this research the amount of reduction noted from the starting test load (CFUs present in the test system) and the amount of resultant microbial ingress into positive test trays was significant. It is recognized that for sterile medical devices the presence of any organisms is not acceptable. However, the real world situation could exist that while microbial ingress may occur in a package, that the quantity of pathogens is insufficient as an infective dose if it is not a zero tolerance pathogen. The concept of infective dose with regard to the aggregate of organisms typically found in a hospital environment is an area warranting future research.

Recommendations For Future Research

There are numerous variables which contribute to the determination of the minimal hole size which may result in microbial ingress in a sterile medical device package. These include:

- Duration of time that the package is exposed to the microbes
- Package Geometry
- Porosity of Package Components
- Thickness of Package Components
- Rigidity of the Package at Test
- Method of Microbial Challenge (Immersion, Aerosol, Dynamic, Static)
- Pressure Differential across the Sterile Barrier
- Types, Sizes, States, and Concentration of the Challenge Organisms
- Types of Package Defects and the Methods of Generating the Defects

This research provides an answer regarding one of these variables, pressure differential. The research indicates that pressure differential is a significant factor contributing to microbial ingress; reference Chapter 5, Results, Table 20. Additional research exploring the other variables is recommended, specifically, exploring the effect of gravitational settling and Brownian Motion as the driving forces on microbial penetration, the effect of static on microbial penetration, examination of additional hole sizes, additional pressure differentials and an exploration of the impaction phenomenon discussed in Chapter 1, Introduction, the impact on the effect of pressure differential under varying rates of inducement, the effect of pressure differential on seal defects, and the impact of temperature variation.

APPENDIX I

APPENDIX I

NEW METHOD DEVELOPMENT

Introduction

For reasons detailed in Chapter 1, Introduction, an alternative means to measure the microbial penetration into packages was needed in order to obtain meaningful results for the primary objective of this research, an examination of the effect of pressure differential on the microbial ingress. In order to employ this new method, aseptically filling packages with sterile agar and then incubating closed packages to see if any microbial growth takes place (Figure 22), it was necessary to verify that the process of filling the package did not significantly influence microbe penetration.

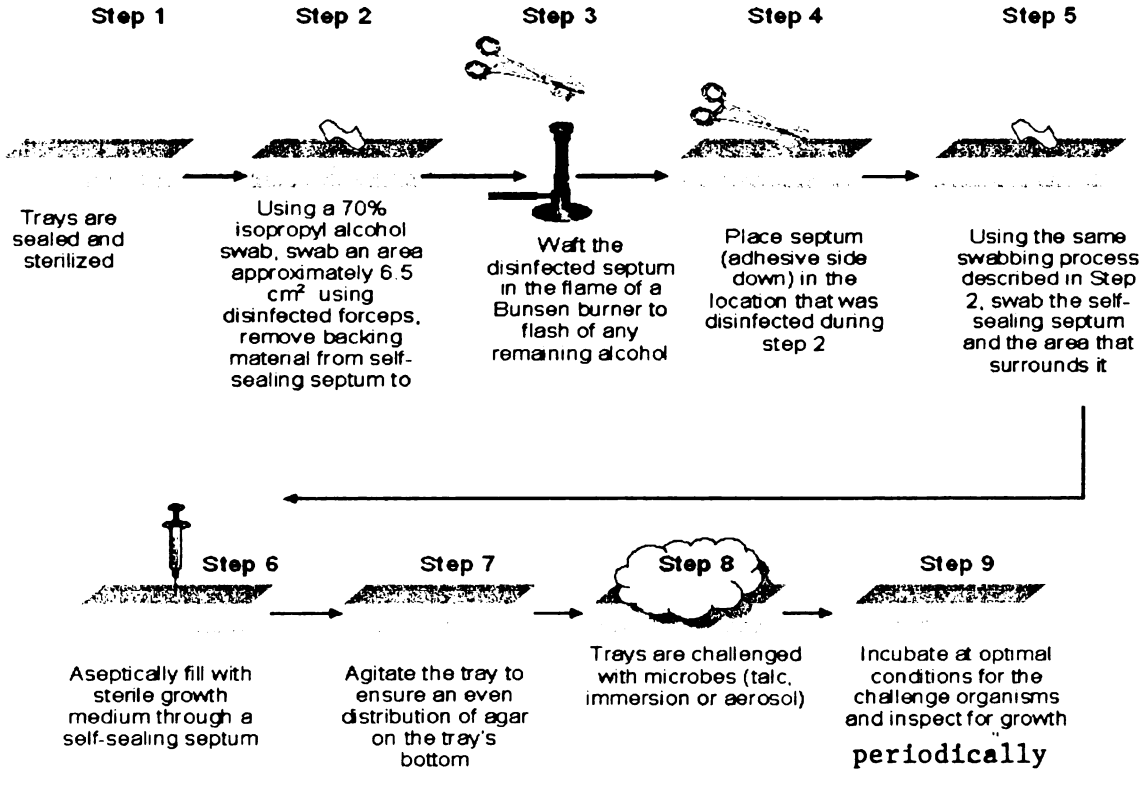


Figure 22 – Proposed Whole Package Microbial Challenge Test through Aseptic Introduction of Sterile Growth Medium

A possible criticism of the new technique is that introduction of sterile agar through the self-sealing septum will result in unwanted contamination and growth and, therefore, false positives. As a result, an experiment was conducted to examine the following working hypothesis:

Working Hypothesis

- Aseptic introduction of the appropriate agar growth medium into a sterile package will not result in unwanted contamination of the package (false positives).

Materials and Methods

Three hundred, eighty uncoated glycolized polyethylene terephthalate (PETG) trays (Figure 23) were sealed with lid stock composed of 1073B Tyvek® with heat seal coating PTH-017 using a SenCorp MD 2420 dual-shuttle heat sealer. Heat sealer settings were 411 K, 517 kPa, with a dwell time of 2.5 seconds, and the trays were thermoformed from stock that had a nominal thickness of 0.0635 cm. Nominal tray dimensions were 16.6624 cm x 12.8524 cm x 2.2225 cm. Sealed trays were sterilized using ethylene oxide (EtO) by Sterigenics, Willowbrook, IL, (EtO cycle 154, prior to test, system run number, SWB_20041007_5268, sterilization date,10/07/04).

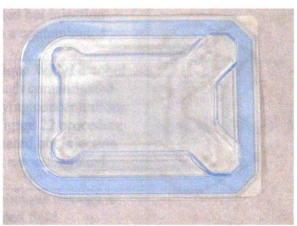


Figure 23 - Uncoated PETG Tray Provided by Perfecseal Sealed with 1073B Heat Seal Coated Tyvek® Provided by Amcor Flexibles

Four test populations were created for the experiment (Table 21). The design of the study was based on the fact that using the traditional procedure (Figure 2); a small random sample was unlikely to contain enough instances of the rare event (contamination) to make reliable inferences. However, a sample size large enough to ensure a reasonable number of rare events to reliably observe was prohibitively expensive. To resolve this situation, trays were subjected to more extreme conditions (injection occurred through a solution containing varying concentrations of microbes-Table 21) than will be present when employing the proposed methodology. In doing so, the methodology became more robust. This allowed sample-to-sample differences in contamination levels to be examined and distinctions to be made between the method and the randomness of nature, as well as to decrease the sample size.

Table 21 - Description of Test Groups

Test Group #	Description	Concentrations Tested (CFU/mL)	Quantity of Trays Challenged
1	Aseptic Technique with NO alcohol swabbing through contaminated fields with varying concentrations of microbes (steps 5 and 8 in Figure 22 are eliminated but injection occurs through a contaminated field)	None, 10^2, 10^4, 10^6, 10^8	150 (30 per concentration)
2	Aseptic Technique WITH alcohol swabbing through contaminated fields with varying concentrations of microbes (Figure 22 procedure step 8 is eliminated but injection occurs through a contaminated field)	None, 10^2, 10^4, 10^6, 10^8	150 (30 per concentration)
3	Full aseptic technique through a non-contaminated field (Figure 22 procedure step 8 is eliminated)	None	30

Table 21 (cont'd)			
Test Group #	Description	Concentrations Tested (CFU/mL)	Quantity of Trays Challenged
4	Inject through varying contaminated fields with no self-sealing septum or aseptic technique (positive control) (steps 2-5 and 8 are eliminated from Figure 22 injection occurs through a contaminated field)	None, 10^2, 10^4, 10^6, 10^8	50 (10 per concentration)

Table 22 - Test Group Procedure Description	
Test Group #	Methodology
1	1. With a 70% Isopropyl Alcohol swab, swab an approximate 6.5 cm^2 area in the center of the lidstock (top) of the tray using moderate pressure for approximately 30 seconds. 2. Using disinfected forceps, pick up a pre-sized piece of septum material and remove the backing material. 3. Dip the septum material in a 70% Isopropyl Alcohol Bath. 4. Waft the disinfected septum in the flame of a Bunsen burner to flash off any remaining alcohol 5. Still using forceps, place the septum material, adhesive side down in the center of the disinfected area of the tray. 6. Firmly press with the forceps to ensure adhesion of the septum material. 7. Using a sterile pipette, transfer 0.5ml of microbe dilution from the stock solution to the center of the septum material. 8. Aseptically draw up approximately 25 mls of equilibrated medium in a sterile syringe using a 16 gauge needle from a test tube. A 16-gauge needle was used for this procedure to facilitate the drawing up of the viscous agar. 9. Replace the 16-gauge needle with a sterile 18-gauge needle. Change to an 18-gauge needle in this step was for two reasons; first to minimize any potential contamination and to minimize the size of the entry port into sterile tray. 10. Inject the entire syringe volume into the tray through the septum. 11. Immediately agitate the tray ensuring distribution of agar on the tray bottom. 12. Allow the medium to completely solidify undisturbed. 13. This process is repeated for each concentration lot of 30 trays. 14. Incubate trays for 72 hours in a controlled environmental chamber set at 37°C +/-2°C; 50% RH. Visually inspect for growth at a time point between 24 and 72 hours. 15. *E. coli* will exhibit growth after 24 hours. After 72 hours the medium will likely be too dry to accurately measure growth.
2	This test group is identical to Test Group One; with the following exception. There is an additional step after Step 7. Prior to the injection of the medium through the septum, the same swabbing process employed in step 1 is employed on the contaminated septum material. Once this is completed, the medium is injected into the tray as described in Test Group One.

Table 22 (cont'd)	
Test Group #	Methodology
3	This group is procedurally identical to Test Group Two with the exception of the steps associated with exposure to microbial contamination. This group was not exposed to any microbial contamination.
4	This group procedurally is identical to Test Group One with the exception that the self-sealing septum material was not used. The sterile medium was injected through the contaminated field directly through the tray lidding material. No effort was made to cover the approximately 1mm hole left in the lidstock from the needle. No aseptic technique employed.

Three test groups and a positive control were examined (Table 21). The first two test groups were grossly contaminated (with a solution of varying concentrations of microbes) on the surface of the package lidstock to create a situation that is more hostile than "normal testing" to provide a worst case environment. The microbial dilutions were added to the injection field prior to the placement of the self-sealing septum for Test Groups 1 and 2 (Table 21). As discussed, injecting through these dilutions allowed for fewer samples to be used.

The third test group represented the proposed test methodology without the microbial challenge (Figure 22, less step 8). Trays were purposefully not contaminated and aseptic filling was conducted after the injection site was swabbed with alcohol.

The fourth group consisted of positive controls. Trays were grossly contaminated, no septum material was used and no aseptic technique alcohol swabbing was done prior to the introduction of the sterile agar. The positive control was included to indicate:

1. The agar can support growth under the conditions of the study
2. The *E. coli* strain used was viable.

The organism used for Test Groups 1, 2, and 4 was *Escherichia coli*, ATCC Number 29181. Media Preparation was performed per standard methods described in Current Protocols in Molecular Biology (Volume 1; Section 1 *ESCHERICHIA COLI*; Media Preparation and Bacteriological Tools; Solid Media Page 1.1.3). The *Escherichia coli* was reconstituted and cultured as described in this same source (Section 1.2; Growth in Liquid Media). The microbe dilutions were prepared as described in this same source (Section 1.3; Growth on Solid Media; Basic Protocol One 'Titering and Isolating Bacterial Colonies by Serial Dilutions'). The Butterfield's stock solution used for the dilutions and the application of the microbes to the test trays was prepared by adding 34 grams of Potassium Phosphate, Monobasic, Crystal (KH_2PO_4); J. T. Baker CAS 7778-77-0; to 500 mls of distilled water. After preparation of the stock solution, the pH was adjusted to 7.2 with 1N Sodium Hydroxide (NaOH). The volume was then brought to 1 liter with distilled water. The solution was then autoclaved for 15 minutes at 121°C.

Sample trays were incubated at 37°C and 50 percent RH in a controlled environmental chamber for no longer than 72 hours. Trays were inspected for growth between 24 hours and 72 hours. Counting of the colony forming units (CFUs) was performed visually using a Quebec Colony Counter. Figure 24 depicts a tray exhibiting

growth. Counting was performed in accordance with the plate counting rules and guidelines described in 'Modern Food Microbiology' (4th Edition).

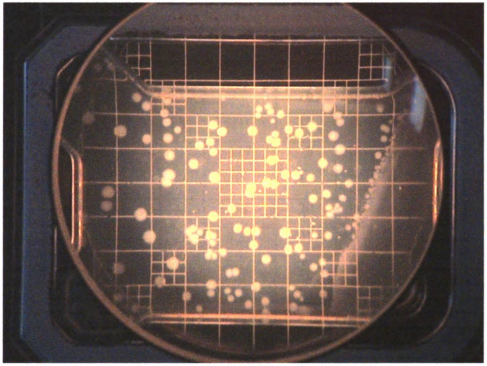


Figure 24 - Tray Exhibiting Growth as Seen Using the Quebec Colony Counter

Results

Table 23 - Test Group 1- Aseptic Technique with NO Alcohol Swabbing Through Contaminated Fields with Varying Concentrations of Microbes (Steps 5 and 8 in Figure 22 are Eliminated but Injection Occurs Through a Contaminated Field)											
Tray	CFU/mL					Tray #	CFU/mL				
***	**0**	**10^2**	**10^4**	**10^6**	**10^8**		**0**	**10^2**	**10^4**	**10^6**	**10^8**
1	0	0	0	0	68	16	0	0	0	1	11
2	0	0	0	3	TNTC*	17	0	0	0	0	121
3	0	0	0	1	177	18	0	0	0	0	22
4	0	0	0	0	0	19	0	0	0	0	136
5	0	0	0	0	78	20	0	0	0	0	93
6	0	0	0	0	125	21	0	0	0	2	0
7	0	0	0	0	TNTC*	22	0	0	0	1	7
8	0	0	0	0	TNTC*	23	0	0	0	0	30
9	0	0	0	0	TNTC*	24	0	0	0	0	1
10	0	0	0	6	3	25	0	0	0	0	TNTC*
11	0	0	0	0	0	26	0	0	0	0	174
12	0	0	0	1	21	27	0	0	0	4	39
13	0	0	0	0	69	28	0	0	0	5	103
14	0	0	0	0	66	29	0	0	0	0	40
15	0	0	0	5	86	30	0	0	0	2	96

***TNTC = Too numerous to count**

*****Incubation Periods:**

0 **58 hours**
10^2 **44.5 hours**
10^4 **33 hours**
10^6 **33 hours**
10^8 **33 hours**

Table 24 - Test Group 2 - Aseptic Technique WITH Alcohol Swabbing Through Contaminated Fields with Varying Concentrations of Microbes (Figure 22 Procedure Step 8 is Eliminated But Injection Occurs Through a Contaminated Field)											
Tray #	CFU/mL					Tray #	CFU/mL				
***	**0**	**10^2**	**10^4**	**10^6**	**10^8**		**0**	**10^2**	**10^4**	**10^6**	**10^8**
1	0	0	0	0	0	16	0	0	0	0	0
2	0	0	0	TNTC*	0	17	0	0	0	0	TNTC*
3	0	0	0	TNTC*	TNTC*	18	0	0	0	0	TNTC*
4	0	0	0	0	0	19	0	0	0	1	0
5	0	0	0	TNTC*	TNTC*	20	0	0	0	TNTC*	0
6	0	0	0	0	TNTC*	21	0	0	0	0	**
7	0	0	0	**	TNTC*	22	0	0	0	0	0
8	0	0	0	0	TNTC*	23	0	0	0	0	0
9	0	0	0	0	TNTC*	24	0	0	0	0	85
10	0	0	0	0	TNTC*	25	0	0	0	4	TNTC*
11	0	0	0	0	TNTC*	26	0	0	0	0	TNTC*
12	0	0	0	0	TNTC*	27	0	0	0	0	0
13	0	**	0	0	0	28	0	0	0	0	TNTC*
14	0	0	0	0	0	29	0	0	0	0	**
15	0	0	0	0	TNTC*	30	0	0	**	**	**

***TNTC=Too numerous to count**
****Sample Not Available-Insufficient Medium**

*****Incubation Periods:**

- **0 40.5 hours**
- **10^2 40.5 hours**
- **10^4 40.5 hours**
- **10^6 41 hours**
- **10^8 41 hours**

Table 25 - Test Group 3 (CONTROL) - Full Aseptic Technique Through a Non-Contaminated Field (Figure 22 Procedure Step 8 is Eliminated)			
Tray #	0 CFU/mL	Tray #	0 CFU/mL
1	0	16	0
2	0	17	0
3	0	18	0
4	0	19	0
5	0	20	0
6	0	21	0
7	0	22	0
8	0	23	0
9	0	24	0
10	0	25	0
11	0	26	0
12	0	27	0
13	0	28	0
14	0	29	0
15	0	30	0

*****Incubation Period: 38.5 hours**

Table 26 - Test Group 4 (POSITIVE CONTROL) - Inject Through Varying Contaminated Fields with no Self-Sealing Septum or Aseptic Technique (Positive Control) (Steps 2-5 and 8 are Eliminated from Figure 22 Injection Occurs Through a Contaminated Field)					
Tray #	CFU/mL				
***	**0**	**10^2**	**10^4**	**10^6**	**10^8**
1	0	0	3	26	TNTC*
2	0	0	0	165	186
3	0	0	0	TNTC*	TNTC*
4	0	0	0	30	TNTC*
5	0	0	0	29	122
6	0	0	0	48	TNTC*
7	0	0	0	24	TNTC*
8	0	0	0	10	TNTC*
9	0	0	0	32	TNTC*
10	0	0	0	123	TNTC*

***TNTC=Too Numerous to Count**

****Incubation Periods:**

0	**27 hours**
10^2	**27 hours**
10^4	**27 hours**
10^6	**27 hours**
10^8	**27 hours**

As established in Chapter 3, Materials and Methods, by introducing the "gross contaminated area," confidence in the methodology can be gained with fewer samples. For each treatment group and each concentration of 10^x, the proportion (rate) of trays exhibiting microbial growth was calculated. Logistic regression was used to model the relationship of rate to the concentration 10^x, for each treatment group. Fisher's exact test was used to assess the statistical significance of group differences at specific concentrations.

Discussion

The regression estimates smooth the raw data that are found in Tables 13, 14, and 16. The tables illustrate that in Groups 1 and 2, no microbial growth was observed at concentrations below 10^6 CFU/mL. In Group 4, no microbial growth was observed at concentrations below 10^4 CFU/mL. This indicates that the contamination threshold lies between a concentration of 10^4 CFU/mL and 10^6 CFU/mL.

At a concentration of 10^6 CFU/mL, the rates of positive trays for the Groups 1 and 2 are 11/30 and 6/28. The one-sided, p-value of the Fisher-Exact Test of this difference is $p = 0.16$ (not below the 0.05 standard for statistical significance). Conclusion: No statistically significant effect from swabbing with alcohol.

At a concentration of 10^8 CFU/mL, the rates of positive trays for the Groups 1 and 2 are 27/30 and 16/27. The one-sided p-value of the Fisher-Exact Test of this difference is $p = 0.008$ (below the 0.05 standard for statistical significance). Conclusion: There is a statistically significant effect from swabbing with alcohol.

In total, there is a statistically significant effect from swabbing with alcohol, ie, employing full aseptic technique (Steps 2-5 of Figure 22) in the expected direction; that is, reducing contamination.

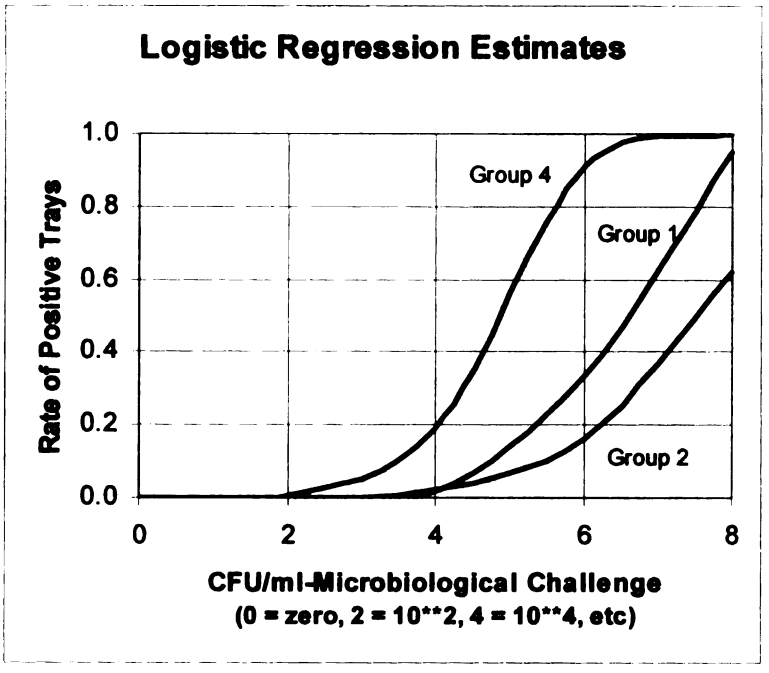


Figure 25 - Logistic Regression Estimates for Groups 1, 2, and 4 (microbial concentrations for Group 3 did not vary)

Conclusion

Working Hypothesis:

- Aseptic introduction of appropriate agar growth medium into a sterile package will not result in unwanted contamination of the package (false positives).

The results indicate that there is no evidence to the contrary regarding Working Hypothesis 1. Therefore, the method does appear to eliminate the shortcomings of the microbial challenge test described in Chapter 1. This method shows promise as a viable alternative to traditional whole-package microbial challenge testing.

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