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Investigation of Environmental Microorganisms Associated with the Intrinsic Microbial Contamination of an Alcohol-Free Mouthwash

Michelle D. Trickett

Investigation of Environmental Microorganisms Associated with the Intrinsic Microbial Contamination of an Alcohol-Free Mouthwash

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Michelle D. Trickett

Dr. Frank Binder, Committee Chairperson
Dr. Marcia Harrison
Dr. Charles Somerville

Marshall University

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Abstract

The United States Pharmacopoeia (USP) Microbial Limits Test and Preservative Effectiveness Test were validated and completed on an alcohol-free mouthwash using standard USP recommended microorganisms and selected organisms isolated from the manufacturing environment. The microbial limits test using environmental isolates was found equivalent to the USP Microbial Limits Test currently used for release of the product. The preservative effectiveness test was performed to determine the efficacy of the parabens system against environmental isolates compared to those recommended in the USP test. In both tests *Pseudomonas aeruginosa* isolates exhibited resistance to the preservative system. All other isolates failed to grow after week one. Temperature studies conducted with environmental isolates to evaluate sanitation procedures for manufacturing indicated organisms exposed to 70˚C for ten minutes resulted in at least a seven log reduction in number. Environmental isolates exposed to 60˚C and 65˚C were found to survive at these elevated temperatures after a ten minute exposure period.

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Literature Review

The fear of intrinsic microbial contamination began in the late 1970's with the discovery of contaminated intravenous products. Also, in the early 1980's the fear increased with the discovery of contaminated povodine-iodine solutions. Products of this nature were never considered for contamination due to their antiseptic or disinfecting properties or the use of a preservative system in the product. With the discovery of this new source of contamination, investigations began into the source and the methods of survival of the contaminating microorganism.

In 1971, Mackel *et al.* conducted an investigation into intrinsically contaminated infusion products that led to an epidemic of septicemia. An investigation into the source of the contamination revealed microorganisms in both of the product manufacturing plants and that the newly introduced screw-cap closures being used were not effective at keeping the product sterile. Along with the epidemic strains of *Enterobacter cloacae* and *Enterobacter agglomerans, Klebsiella pneumoniae, Acinetobacter calcoaceticus, Bacillus species, Pseudomonas* species, *Planococcus* species, *Corynebacterium* species, and *Staphylococcus epidermidis* were also found at different locations throughout the manufacturing plants. Due to the ability of moisture from autoclaving procedures to penetrate the new screw-cap and elastomer closures on the product bottles, contamination of the intravenous fluid could occur easily when used repetitively within the hospital setting. (Mackel *et al.*, 1975)

In 1980, Craven *et al.* conducted an investigation into an outbreak of pseudobacteremia due to contamination of a povidone-iodine solution. The solution was found to be intrinsically contaminated with *Pseudomonas cepacia* from the manufacturing process (Craven *et al.*, 1981). In 1988 another outbreak of bacteremia and pseudobacteremia due to *P. cepacia* was discovered

and linked to a blood gas analyzer at the Clinical Center of the National Institutes of Health's clinical intensive care unit (Henderson *et al.*, 1988).

In 1983 Berkelman *et al*. conducted an investigation of a manufacturing plant after reports of intrinsic contamination of an iodophor solution. In their study a plant inspection was done along with scanning electron microscopy (SEM) of polyvinyl chloride (PVC) sections of pipe removed from the manufacturing plant. A batch of the Prepodyne solution was made in the laboratory using naturally contaminated water. Storage of the product was also replicated in the laboratory. In their plant inspection, it was found that the water used to manufacture the product was not filtered or treated before mixing, but in some cases it was heated. The pipes, made of steel, copper, PVC, or cast iron, were cleaned only between batches of the product. The records of the plant showed that products found to be contaminated were stored before packaging for five to eighteen days in the contaminated pipes. This information was key after the scanning electron microscopy of the interior surfaces of the PVC pipes showed large numbers of bacterial growth. Figure 1 (modified from Berkelman *et al.,* 1983) shows a SEM of the interior of one of the PVC pipe sections recovered from the manufacturing facility. In their experiments they were unable to recover bacteria from the Prepodyne solution made in the laboratory or from Prepodyne solution that had been incubated for less than 48 hours in sections of the contaminated PVC pipe. However, Prepodyne solution incubated in the sections of PVC pipe for 48 and 72 hours contained *Pseudomonas aeruginosa*. The authors determined that the incubation of the contaminated iodophor solution from this manufacturing plant was due to the extended period of time the product was held in the contaminated PVC pipes before packaging. (Berkelman *et al*., 1983)

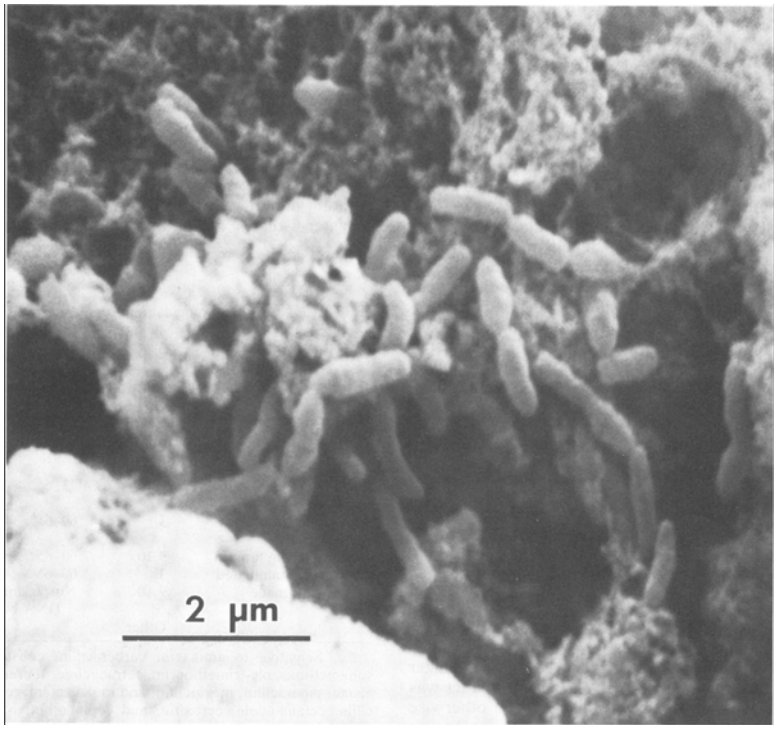

Figure 1. Scanning electron micrograph of the interior surface of a plant PVC pipe used in the production of the contaminated iodophor solution investigated by Berkelman *et al*. (Berkelman *et al*., 1983).

Anderson and Berkelman extended the study of the previously mentioned contaminated iodophor solution to understand the survival of the *P. aeruginosa* in the solution and possible methods of resistance to the iodophor solution. In this study the solution was challenged with high numbers of the *P. aeruginosa,* and survival was determined during each step of the manufacturing process. Further testing of the PVC pipe was also done to determine the survival rate of *P. aeruginosa*. Anderson *et al.* found that during the production steps of the solution, *P. aeruginosa* did not survive after the final step indicating no resistance to the iodophor was induced during the separate steps of production. Again *P. aeruginosa* was recovered from a 1:4 dilution of solution incubated in sections of contaminated PVC pipe for up to 168 days. Although a method of resistance was not determined from these experiments, a method was established for

further work in this area, and more questions about product safety were raised. (Anderson *et al*., 1983)

An investigation of an intrinsically contaminated respiratory therapy solution began after infants in a special care nursery showed positive cultures of *Pseudomonas pickettii.* An investigation of the production facility recovered *P. pickettii* cultures from four separate locations in the plant. The contaminated lots were formulated with water that was not passed through an 83˚C holding tank. The temperature within this holding tank would have been sufficient to kill the contaminating strain in the respiratory therapy solution according to McNeil *et al*. This product was voluntarily recalled after the contamination was discovered. (McNeil *et al.*, 1985)

In 1985, another intrinsically contaminated commercial respiratory therapy solution sparked an investigation into the survival of *Pseudomonas pickettii* under varying environmental conditions. Anderson *et al.* studied *P. pickettii* for heat tolerance, growth at temperature between 15˚C and 42˚C in the saline solution, and also, growth in small and large volumes of the product. The results indicated that *P. pickettii* was able to grow well at temperatures between 22˚C and 42˚C but exhibited slower growth at 15˚C. When exposed to elevated temperatures in a water bath at 50˚, 55˚, 60˚, *P. pickettii* had a D-value of 26.0, 1.9, and 0.7 minutes respectively. The D-value represents the time in minutes required at the given temperatures to reduce the surviving population of microorganisms 10-fold. When inoculated in a small or large volume of saline solution, *P. pickettii* proliferated for 168 hours to a density of 10^6 colony forming units (cfu)/ml. (Anderson *et al.*, 1985)

In 1989, cases of *Pseudomonas cepacia* were found in Texas hospital patients who had received peritoneal dialysis treatment. The infections were linked to a Clinidine povidone-iodine

solution being used within the hospital. Cultures of *P. cepacia* were recovered from three unopened containers of the solution. An investigation of the manufacturing facility was conducted, and the product was voluntarily recalled by the company (CDC, 1989).

Anderson *et al.,*. also, conducted a study to evaluate the prolonged survival of *Pseudomonas cepacia* in a povidine-iodine solution in 1990. Samples were taken weekly, and survival of *P. cepacia* occurred during twenty-nine weeks of sampling. This was documented as sixty-eight weeks after the actual manufacturing of the PI solution. Anderson *et al.* attributed this survival and fluctuating growth densities to *P. cepacia* cells being released from the extra-cellular matrix over time. (Anderson *et al.*, 1990).

In 1990, an antimicrobial solution was investigated for contamination with *Mycobacterium gordonae* due to improper water sterilization. Quality control procedures were reevaluated after this investigation and further testing was done to avoid continuous problems with intrinsic contamination. (Tokars *et al.*, 1990)

In 1998, the CDC reported the voluntary recall of Kentron Alcohol Free Mouthwash and Gargle due to intrinsic contamination with *Burkholderia cepacia* (formerly *Pseudomonas cepacia*). Patients from two Arizona hospitals tested positive for *B. cepacia* after cultures were taken from their respiratory tracts while intubated or ventilated in the hospitals' intensive care units. During the time the patients were in the ICU, they received oral care with the Kentron mouthwash. In the investigation of the contaminated mouthwash, *B. cepacia, Alcaligenes xylosoxidan,* and a member of the *Pseudomonas fluorescens* group were recovered from unopened bottles. (*JAMA*, 1999) Research into the method and source of the contamination, along with the method of survival of microorganisms in alcohol-free mouthwashes, merits further investigation and research.

Introduction

Each year consumers are informed of thousands of products that are recalled for varying reasons. These products can include food, pharmaceutical products, pet products, children's toys, beauty products and others. Each one poses a threat to the consumer and the producer of the product in some way. Not only do the companies suffer financial loss, as well as, litigation; but serious and severe side effects can occur if the product reaches the consumer or a clinical setting.

A serious problem with pharmaceutical products can occur during production. Intrinsic microbial contamination occurs before the product reaches the consumer and could be from the incoming water, raw materials, manufacturing equipment, or sanitation procedures used in manufacturing the product. Unfortunately, not all contaminated products are discovered before they reach the public. In 1998, Alcohol Free Mouthwash and gargle manufactured by Kentron was found to be intrinsically contaminated with *Burkholderia cepacia* after two Arizona hospitals reported positive cultures for the microorganism. All patients that tested positive had been intubated, ventilated, and received oral care with the Kentron mouthwash. Kentron voluntarily recalled this mouthwash product (CDC, 1998). In 2005, a Medline Industries, Inc alcohol-free mouthwash was also voluntarily recalled after suspected *B. cepacia* contamination. Again patients that had been intubated and received oral care with the mouthwash prior to onset of pneumonia tested positive for *B. cepacia* (CDC, 2005).

The mouthwash used in this study is also an alcohol-free mouthwash that contains a parabens preservative system. Parabens or *p*-hydroxybenzoic acid esters can include methyl, ethyl, propyl, butyl, or benzyl parabens or several of these in combination for increased preservative effectiveness. Parabens are effective against fungi but less effective against bacteria. Parabens preservative systems are also less effective against *Pseudomonas* species (Hugo and

Russel, 1983). The alcohol-free mouthwash used in this study utilized a parabens preservative system, was sugar-free, and packaged in four and eight ounce plastic bottles. During the manufacture of this product, incoming water is chlorinated, run through activated charcoal, and then run through two sets of ion exchange resins. The water then passes through two sets of ultraviolet lights and two sets of 0.45 µm filters. The water is then put in mixing kettles through stainless steel pipes where the raw materials are added and mixed. After the mouthwash is formulated, it is run through stainless steel pipes to the packaging system to be placed in plastic bottles. After production of the mouthwash, the mixing kettles are then cleaned with water through garden hoses with nozzles. These hoses are stored hanging on the wall in various rooms of the manufacturing plant. This practice unfortunately is believed to have caused the intrinsic contamination of the mouthwash due to bacterial biofilm accumulation in the hoses and hose nozzles. Test results from a microbial limits test, conducted prior to the start of this study, showed contamination with a *Pseudomonas aeruginosa*. Inspection of incoming water showed no contamination, and further investigation of the source was conducted. Inspection of the manufacturing equipment and sanitation equipment revealed biofilms. The environmental isolates used in this study were recovered from the garden hoses and nozzles used in the various rooms of the plant for sanitation.

One objective of this study was to compare the United States Pharmacopeia (USP) Microbial Limits test using the standard USP organisms and the environmental isolates recovered at the manufacturing facility. The Microbial Limits Test is the most widely used test for the release of pharmaceuticals. It includes two parts: the estimation of aerobic microbial count and the demonstration of freedom from the selected microorganisms.

A second objective was to evaluate the preservative system used in the mouthwash by comparing the standard United States Pharmacopeia (USP) organisms and the isolates recovered from the manufacturing environment.

A third objective was to evaluate the survival of the environmental isolates when exposed to temperature between 60˚C and 70˚C in order to improve the manufacturer's ability to control further intrinsic microbial contamination.

Materials and Methods

Isolation of Environmental Organisms

Garden hoses and their nozzles used in the rinsing of the product formulation tanks were the source of the environmental isolates used in this study. A paraffin square was placed on one end of a 12 inch piece of hose or a nozzle and filled with peptone tween-80 solution (Peptone Tween-80 was prepared by adding 1g peptone and 1mL Tween-80 to 1 L of distilled water. This medium was autoclaved for 15 minutes at 123˚C) (Difco, Franklin Lakes, NJ). Another square of paraffin was placed on the other end of the hose or nozzle, and the contents were shaken to recover the isolates. The peptone tween-80 solution was removed and plated on a tryptic soy recovery media (TSA was prepared by adding 30 g TSB and 15 g agar to 1 L of distilled water. This medium was autoclaved for 15 minutes at 123˚C.) (Difco) to isolate the dominate organisms that were then used in this study. The isolates used were recovered from the following room's hoses at the manufacturing facility:

Isolate 1: Garden hose cleaned with isopropyl alcohol from room 2 (*Pseudmonas aeruginosa 1*)

Isolate 2: Unclean garden hose nozzle from room 3 (*P. aeruginosa 2*)

Isolate 3: Garden hose cleaned with isopropyl alcohol from room 4 (*Pseudomonas* species)

Microbial Limits Test (The United States Pharmacopeia, USP 30/NF 25)

The USP Microbial Limits Test is the most widely used test to assess the microbiological quality of a manufactured pharmaceutical. The test has two components. The first is to assess the number of aerobic microorganisms present in a finished manufactured product, and the second is to certify the sample is free from the selected organisms. Validation of this test method is required for each pharmaceutical product before this procedure can be utilized.

Preparation of culture media and dispersing solution

1. Tryptic Soy Broth (TSB) (Difco)– TSB was prepared by adding 30 g TSB to 1 L of distilled water. This medium was then dispensed into sterile culture tubes (10 ml/tube) and then sterilized for 15 minutes at 123˚C.
2. Tryptic Soy Agar (TSA) (Difco) – TSA was prepared by adding 30 g TSB and 15 g agar to 1 L of distilled water. This medium was autoclaved for 15 minutes at 123˚C and dispersed into 100x15 mm petri dishes (25 ml/plate).
3. Peptone Tween-80 (Difco) –Peptone Tween-80 was prepared by adding 1 g peptone and 1mL Tween-80 to 1 L of distilled water. This medium was dispersed into sterile media bottles (90 ml/bottle) and autoclaved for 15 minutes at 123˚C.
4. D/E Neutralizing Agar (Dey and Engley) (Difco) – D/E was prepared by adding 39 g D/E broth and 15 g of agar to 1 L of distilled water. This medium was autoclaved for 15 minutes at 123˚C and dispersed into 100 x 15 mm petri dishes (25 ml/plate).

5. Letheen Broth (Difco) – Letheen broth was prepared by adding 25.7 g letheen broth to 1 L distilled water. This medium was dispersed into sterile media bottles (90 ml/bottle) and autoclaved for 15 minutes at 123˚C.
6. Selective Media:
    a. Pseudomonas Isolation Agar (PIA) (Difco) used in the detection of *Psuedomonas aeruginosa* was prepared by adding 4.5 g PIA and 2ml glycerin to 98 ml of distilled water.
    b. Mannitol Salt Agar (MSA) (Difco) used for the detection of *Staphlococcus aureus* was prepared by adding 11.1 g MSA to 100 ml of distilled water.
    c. MacConkey Agar (Mac) (Difco) used for the detection of *Escherichia coli* was prepared by adding 5.0 g Mac to 100 ml distilled water.
    d. Salmonella Shigella Agar (SS) (Difco) used for the detection of *Salmonella typhimurium* was prepared by adding 6.0 g SS to 100 ml of sterilized distilled water. This media was not autoclaved.

Unless otherwise indicated, each of the selective media was then sterilized at 123˚C for 15 minutes and dispensed into 18 x 150 mm culture tubes (10 ml/tube) and then slanted.

Preparation of Inoculum

Four organisms, *Pseudomonas aeroginosa (ATCC #27853), Staphylococcus aureus (ATCC #25923), Escherichia coli (ATCC #25922), and Salmonella typhimurium (ATCC #14028),* were individually streaked on TSA medium and grown overnight at 35ºC. Cell suspensions for each organism were prepared in TSB and diluted to $1:10^{-6}$ to achieve a low inoculum level (< 100 cfu). Dilutions ($1:10^{-5}$, $1:10^{-6}$, and $1:10^{-7}$) were also plated on TSA to

determine the initial inoculum density. These plates were grown overnight at 35ºC and cfu were counted.

The environmental isolates used in this study were streaked on TSA medium and grown overnight at 35ºC. A cell suspension was prepared in TSB for each isolate, and dilutions were made as described previously. Dilutions ($1:10^{-5}$, $1:10^{-6}$, and $1:10^{-7}$) were, also, plated on TSA to determine the initial inoculum level. These organisms were grown overnight at 35ºC and cfu were counted.

Sample Preparation

The sample preparation (1:10 dilution) was prepared by transferring the alcohol-free mouthwash (10 ml) into a Peptone Tween-80 dispersing solution (90 ml). This sample was then aseptically dispersed into sterile 18 x 150 mm culture tubes (10 ml/ tube.)

Validation of the Test Procedure

To estimate the number of viable aerobic microorganisms in the test sample, four sterile tubes were inoculated with 100 µL of a $1:10^{-6}$ dilution of the designated standard organisms and vortexed to mix the sample. The entire contents of each tube were then passed through separate 0.45 µm membrane filters (Nalgene, Rochester, NY). Each filter was then transferred to the surface of a D/E neutralizing agar medium and incubated for 48 hours at 35ºC. One sample remained uninoculated as a control. The number of recovered organisms was assessed by counting the cfu that developed on the surface of each filter after 48 hours. The objective of this component of the Microbial Limits Test was to demonstrate that the indicator organisms could be recovered when present in low numbers.

To demonstrate that the test product was free from the selected organisms, four sterile tubes were inoculated with 100 µL of the $1:10^{-6}$ dilution of each designated organism. Each tube was then vortexed, and the entire contents passed through separate 0.45 µm membrane filters (Nalgene). Each filter was then transferred to a letheen broth enrichment medium (100 ml). An additional sample remained as an uninoculated control. Samples were incubated at 35ºC for 24 hours and then observed for turbidity. One loopful (10 µL) from each inoculated sample was then transferred to the appropriate selective medium and incubated at 35ºC for 24 hours. The uninoculated control sample was sub-cultured onto all four selective media.

This procedure was repeated as described above except environmental isolates replaced the USP designated isolates. All experiments were completed in duplicate.

Preservative Effectiveness Test (The United States Pharmacopeia, USP 30/NF 25)

The Preservative Effectiveness Test is a standard test used to determine the efficacy of a preservative system used in the manufacture of a pharmaceutical product. In this test the product is inoculated with a high density of selected microorganisms. The USP guidelines stipulate that bacteria should have no less than a 1.0 log reduction by day fourteen of the test and no increase from the count at fourteen days. Yeasts should have no increase from the initial count at fourteen days and twenty-eight days (USP, 2006).

Preparation of Culture Media

1. Tryptic Soy Broth (TSB) (Difco) - TSB was prepared by adding 30 g TSB to 1 L of distilled water that was dispensed into sterile culture tubes (10 ml/tube) and then sterilized for 15 minutes at 123˚C.
2. Tryptic Soy Agar (TSA) (Difco) – TSA was prepared by adding 30 g TSB and 15 g agar to 1 L of distilled water. This medium was autoclaved for 15 minutes at 123˚C and dispersed into 100 x 15 mm petri dishes (25 ml/plate).
3. Sabouraud Dextrose Agar (SDA) (Difco) – SDA was prepared by adding 30 g of SDA to 1 L of distilled water. This medium was autoclaved for 15 minutes at 123˚C and dispersed into 100 x 15mm petri dishes (25 ml/plate).
4. Peptone Tween-80 (Difco) – Peptone Tween-80 was prepared by adding 1 g peptone and 1 ml tween 80 to 1 L of distilled water. This medium was dispersed into sterile media bottles and autoclaved for 15 minutes at 123˚C.

5. D/E Neutralizing Agar (Difco) – D/E was prepared by adding 39 g D/E broth and 15 g of agar to 1 L of distilled water. This medium was autoclaved for 15 minutes at 123˚C and dispersed into 100 x 15 mm petri dishes (25 ml/plate).
6. Saline solution (Hyclone, Logan, UT)– Saline solution was prepared by adding 100 ml saline solution to 900 ml distilled water. This medium was then sterilized for 15 minutes at 123˚C.

Preparation of Inoculum

Pseudomonas aeruginosa (ATCC #9027), *Staphylococcus aureus* (ATCC #6538), *Escherichia coli* (ATCC #8739), *Candida albicans* (ATCC #10231) and *Aspergillus niger* (ATCC #16404), were grown overnight from stock culture in 10 ml TSB medium. Following incubation, *Psuedomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* were subcultured onto TSA and grown at 35ºC for 24 hours prior to testing. *Candida albicans* was grown at 25ºC on TSA for five days prior to experimentation. *Aspergillus niger* was subcultured onto SDA medium and incubated at 25ºC for seven days prior to testing. Suspensions of all organisms were prepared in sterile saline, and dilutions were prepared using the standard plate-count method to estimate the original inoculum density.

The environmental isolates were grown on TSA overnight at 35ºC and a suspension of each isolate was then prepared in saline. Dilutions were completed to estimate initial inoculum densities using the standard plate-count method.

Procedure

Alcohol-free mouthwash (20 ml) was placed in each of five sterile media bottles and inoculated with 100 µL of the prescribed test organism containing 10^5 to 10^6 cfu/ml sample. All cultures were then incubated at 25ºC. Following incubation at 7, 14, 21, and 28 days, aliquots (1 ml) of the mouthwash were removed and added to 9 ml of peptone tween-80 in sterile test tubes. These samples were then passed through separate 0.45 µm membrane filters (Nalgene) and placed on D/E recovery medium. The recovery media inoculated with *Pseudomonas aeruginosa*, *Staphylococcu , aureus,* and *Escherichia coli* were incubated at 35ºC for 48 hours. *Candida albicans* and *Aspergillus niger* were incubated at 25ºC for seven days. Survival of *P. aeruginosa* isolates in the test product required dilutions (1:10 and 1:100) at each sampling time.

This test procedure was repeated by replacing the USP designated organisms with the environmental isolates recovered from the manufacturing environment. All tests were completed in duplicate.

Effect of Elevated Temperatures on Survival of Environmental Isolates

Temperature studies were done to assess the ability of the environmental isolates to survive at elevated temperatures between 60˚C and 70˚C. These studies were undertaken to help the manufacturer control further intrinsic microbial contamination using heat sanitization.

Preparation of culture media

1. Tryptic Soy Broth (TSB) (Difco) – TSB was prepared by adding 30 g TSB to 1 L of distilled water that was dispensed into sterile culture tubes (10 ml/tube) and then sterilized for 15 minutes at 123˚C.
2. Tryptic Soy Agar (TSA) (Difco)– TSA was prepared by adding 30 g TSB and 15 g agar to 1 L of distilled water. This medium was autoclaved for 15 minutes at 123˚C and dispersed into 100 x 15 mm petri dishes (25 ml/plate).
3. Saline solution (Hyclone) – Saline solution was prepared by adding 100 ml saline solution to 900 ml distilled water. This medium was then sterilized for 15 minutes at 123˚C.

Preparation of Inoculum

The environmental isolates were grown overnight on TSA at 35˚C. A cell suspension was made in TSB, and dilutions were made to determine initial inoculum densities by the standard plate count method.

Test Procedure

An aliquot (100 µL) of the original suspension was added to TSB (10 ml) tempered to 60˚C. An aliquot (1 ml) was removed at one, five, and ten minute intervals and placed in 10 ml TSB. Samples were then filtered through separate 0.45 µm membrane filters (Nalgene) and placed on surface of tryptic soy agar medium. Cultures were then incubated at 35˚C for 48 hours. The number of recovered organisms was assessed by counting the number of cfu on the surface of each membrane filter. This test procedure was repeated for 65˚C and 70˚C and completed in duplicate for each isolate and temperature. This procedure was also completed replacing the tryptic soy broth with saline using exposure times of five and ten minutes.

Results

United States Pharmacopeia Microbial Limits Test

The microbiological quality of the alcohol-free mouthwash was assessed using the standard USP Microbial Limits Test. The validation of the aerobic plate count method results are presented in Tables 1 and 3. The results for the validation that the product was free from the USP designated organisms are presented in Tables 2 and 4. The standard USP methods were validated, and all designated organisms were recoverable when present in low numbers. The mouthwash was also certified as free from the designated USP organisms.

Table 1. Validation of the USP Microbial Limits Test with Alcohol-Free Mouthwash, Lot 21021A. Aerobic Plate Count (Replicate 1).

Organism	Initial Inoculum cfu/100 µL	Recovery on D&E Agar 48hrs at 35˚C (cfu/mL)
Pseudomonas aeroginosa (ATCC #27853)	15	13
Staphylococcus aureus (ATCC #25923)	63	52
Escherichia col I (ATCC #25922)	70	55
Salmonella typhimurium (ATCC #14028)	69	70
Uninoculated Control	N/A	<10

Table 2. Validation of the Microbial Limits Test with Alcohol-Free Mouthwash, Lot 21021A. Freedom From Selected Organisms (Replicate 1).

Organism	Selective Medium	+ Controls	Growth in Letheen	Test Sample (Uninoculated)
Pseudomonas aeruginosa	PIA	+	+	-
Staphylococcus aereus	MS	+	+	-
Escherichia coli	MAC	+	+	-
Salmonella typhimurium	SS	+	+	-

Table 3. Validation of the USP Microbial Limits Test with Alcohol-Free Mouthwash, Lot 20475F. Aerobic Plate Count (Replicate 2).

Organism	Initial Inoculum cfu/100 µL	Recovery on D&E Agar 48hrs at 35˚C (cfu/ml)
Pseudomonas aeroginosa (ATCC #27853)	140	13
Staphylococcus aureus (ATCC #25923)	165	52
Escherichia coli (ATCC #25922)	107	55
Salmonella typhimurium (ATCC #14028)	123	70
Uninoculated Control	N/A	<10

Table 4. Validation of the Microbial Limits Test with Alcohol-Free Mouthwash, Lot 20475F. Freedom From Selected Organisms (Replicate 2).

Organism	Selective Medium	+ Controls	Growth in Letheen	Test Sample (Uninoculated)
Pseudomonas aeruginosa	PIA	+	+	-
Staphylococcus aereus	MS	+	+	-
Escherichia coli	MAC	+	+	-
Salmonella typhimurium	SS	+	+	-

Microbial Limits Test Using Environmental Isolates

The microbiological quality of the mouthwash was also assessed by modifying the Microbial Limits Test with the selected environmental isolates. The results are shown in Tables 5 through 8. The standard USP method was validated for use with the environmental isolates, and organisms were recoverable when present in low numbers. The mouthwash was also certified free of the selected environmental isolates.

Table 5. Validation of the USP Microbial Limits Test with Alcohol-Free Mouthwash, Lot 21021A, using selected environmental isolates. Aerobic Plate Count. (Replicate 1).

Organism	Initial Inoculum cfu/100 µL	Recovery on D&E Agar 48hrs at 35˚C (cfu/ml)
Pseudomonas aeruginosa 1	96	90
Pseudomonas aeruginosa 2	59	50
Pseudomonas sp.	66	77
Uninoculated Control	N/A	<10

Table 6. Validation of the USP Microbial Limits Test with Alcohol-Free Mouthwash, Lot 21021A, using selected environmental isolates. Freedom From Selected Organisms (Replicate 1).

Organism	Selective Medium	+ Controls	Growth in Letheen	Test Sample (Uninoculated)
P. aeruginosa 1	PIA	+	+	-
P. aeruginosa 2	PIA	+	+	-
Pseudomonas sp.	MAC	+	+	-

Table 7. Validation of the USP Microbial Limits Test with Alcohol-Free Mouthwash, Lot 21021A, using selected environmental isolates. Aerobic Plate Count. (Replicate 2).

Organism	Initial Inoculum cfu/100 µL	Recovery on D&E Agar 48hrs at 35˚C (cfu/ml)
Pseudomonas aeruginosa 1	150	141
Pseudomonas aeruginosa 2	38	40
Pseudomonas sp.	16	20
Uninoculated Control	N/A	<10

Table 8. Validation of the USP Microbial Limits Test with Alcohol-Free Mouthwash, Lot 21021A, using selected environmental isolates. Freedom From Selected Organisms (Replicate 2).

Organism	Selective Medium	+ Controls	Growth in Letheen	Test Sample (Uninoculated)
P. aeruginosa 1	PIA	+	+	-
P. aeruginosa 2	MSA	+	+	-
Pseudomonas sp.	MAC	+	+ (after 48 hrs)	-

United States Pharmacopeia Preservative Effectiveness Test Results

The preservative capacity of the alcohol-free mouthwash was assessed using the standard USP Preservative Effectiveness Test. In both USP preservative tests, *Pseudomonas aeruginosa* showed resistance to the parabens preservative system. At least a 1 log reduction from the initial inoculum density was observed in both tests by week 1. However, this organism was recoverable after four weeks in the test product. *Staphlococcus aureus, Escherichia coli, Candida albicans,* and *Aspergillus niger* were not recovered from the inoculated mouthwash after one week incubation in the test product. (Tables 9 and 10)

Table 9. United States Pharmacopeia Preservative Effectiveness Test with Alcohol-Free Mouthwash, Lot 20475A. (Replicate 1).

		Week 1	Week 2	Week 3	Week 4
Organism	Initial Inoculum (cfu/ml)	(cfu/ml recovered on D/E agar grown at 35ºC)			
Pseudomonas aeruginosa (ATCC #9027)	3.40×10^5	3.04×10^3	4.96×10^3	2.03×10^3	1.48×10^3
Staphylococcus aureus (ATCC #6538)	9.05×10^5	0[a]	0	0	0
Escherichia coli (ATCC #8739)	8.00×10^5	0	0	0	0
Candida albicans (ATCC #10231)	7.50×10^4	0	0	0	0
Aspergillus niger (ATCC #16404)	2.50×10^4	0	0	0	0

[a]0 indicates <20 cfu/ml

Table 10. United States Pharmacopeia Preservative Effectiveness Test with Alcohol-Free Mouthwash, Lot 20475A. (Replicate 2).

		Week 1	Week 2	Week 3	Week 4
Organism	Initial Inoculum (cfu/ml)	(cfu/ml recovered on D/E agar grown at 35ºC)			
Pseudomonas aeruginosa (ATCC #9027)	3.45×10^5	9.08×10^3	2.85×10^3	5.90×10^3	1.10×10^4
Staphylococcus aureus (ATCC #6538)	4.00×10^4	0[a]	0	0	0
Escherichia coli (ATCC #8739)	3.20×10^5	0	0	0	0
Candida albicans (ATCC #10231)	1.05×10^6	0	0	0	0
Aspergillus niger (ATCC #16404)	3.50×10^4	0	0	0	0

[a]0 indicates <20 cfu/ml

Environmental Preservative Effectiveness Test Results

The preservative capacity of the alcohol-free mouthwash was also assessed using the environmental organisms isolated from the manufacturing facility. *Pseudomonas aeruginosa* isolates 1 and 2 showed resistance to the parabens preservative system in both environmental preservative tests. At least a 1 log reduction from the initial inoculum density was observed for *P. aeruginosa 1* while a 2 log reduction from the initial inoculum density was observed for *P. aeruginosa 2. Isolate 3*, designated as *Pseudomonas* species, was not recoverable as of week 1. (Tables 11 and 12)

Table 11. United States Pharmacopeia Preservative Effectiveness Test with Alcohol-Free Mouthwash, Lot 20475C, using selected environmental isolates. (Replicate 1).

	Number of Surviving Cells (cfu/ml product)[a]		
Time	*P. aeruginosa 1*	*P. aeruginosa 2*	*Pseudomonas* species
Initial Time	4.75×10^6	1.08×10^6	2.35×10^6
Week 1	TNTC[b]	TNTC	0[c]
Week 2	4.1×10^3	TNTC	0
Week 3	TNTC	3.24×10^3	0
Week 5	5.00×10^4	1.89×10^3	0

[a]colony forming units recovered on D/E Neutralizing Agar after 48 hours at 35˚C.
[b]TNTC indicates Too Numerous To Count (>300/ml)
[c]0 indicates <20 cfu/ml

Table12. United States Pharmacopeia Preservative Effectiveness Test with Alcohol-Free Mouthwash, Lot 20475C, using selected environmental isolates. (Replicate 2).

	Number of Surviving Cells (cfu/ml product)[a]		
Time	*P. aeruginosa 1*	*P. aeruginosa 2*	*Pseudomonas* species
Initial Time	4.80×10^6	2.95×10^6	3.30×10^6
Week 1	7.68×10^4	9.52×10^4	0[b]
Week 2	6.76×10^4	6.92×10^4	0
Week 3	6.12×10^4	9.76×10^4	0
Week 5	6.76×10^4	5.20×10^4	0

[a]colony forming units recovered on D/E Neutralizing Agar after 48 hours at 35˚C.
[b]0 indicates <20 cfu/ml

Temperature versus Exposure Time Using Tryptic Soy Broth:

The effect of elevated temperatures on environmental isolates in tryptic soy broth was assessed at 60˚C, 65˚C, and 70˚C. The results indicate that exposure for 10 minutes at 70˚C results in at least a 7 log reduction in number for the environmental isolates when tested in tryptic soy broth. (Tables 14 through 19)

Table 14. Effect of elevated temperature on the survival of *Pseudomonas aeruginosa 1* in tryptic soy broth (Replicate 1).

		Number of Surviving Cells (cfu/ml)			
Temperature (˚C)	Initial Time	1 min	5 min	10 min	Log reduction @ 10 min
60	9.6×10^7	TNTC[a]	77	0[b]	7.982
65	9.6×10^7	26	0	0	7.982
70	9.6×10^7	0	0	0	7.982

[a]TNTC indicates Too Numerous To Count
[b] 0 indicates <10 cfu/ml

Table 15. Effect of elevated temperature on the survival of *Pseudomonas aeruginosa 1* in tryptic soy broth (Replicate 2)

		Number of Surviving Cells (cfu/ml)			
Temperature (˚C)	Initial Time	1 min	5 min	10 min	Log reduction @ 10 min
60	1.24×10^8	TNTC[a]	16	17	6.863
65	1.24×10^8	TNTC	TNTC	0[b]	8.093
70	1.24×10^8	0	0	0	8.093

[a]TNTC indicates Too Numerous To Count
[b] 0 indicates <10 cfu/ml

Table 16. Effect of elevated temperature on the survival of *Pseudomonas aeruginosa 2* in tryptic soy broth (Replicate 1)

		Number of Surviving Cells (cfu/ml)			
Temperature (˚C)	Initial Time	1 min	5 min	10 min	Log reduction @ 10 min
60	6.8×10^7	28	0[a]	0	7.833
65	6.8×10^7	29	0	0	7.833
70	6.8×10^7	0	0	0	7.833

[a] 0 indicates <10 cfu/ml

Table 17. Effect of elevated temperature on the survival of *Pseudomonas aeruginosa 2* in tryptic soy broth (Replicate 2).

		Number of Surviving Cells (cfu/ml)			
Temperature (˚C)	Initial Time	1 min	5 min	10 min	Log reduction @ 10 min
60	8.5×10^7	TNTC[a]	TNTC	TNTC	N/A
65	8.5×10^7	0[b]	0	0	7.929
70	8.5×10^7	0	0	0	7.929

[a]TNTC indicates Too Numerous To Count
[b] 0 indicates <10 cfu/ml

Table 18. Effect of elevated temperature on the survival of the *Pseudomonas* species in tryptic soy broth (Replicate 1).

		Number of Surviving Cells (cfu/ml)			
Temperature (˚C)	Initial Time	1 min	5 min	10 min	Log reduction @ 10 min
60	1.26×10^7	TNTC[a]	TNTC	61	5.315
65	1.26×10^7	TNTC	0[b]	0	7.100
70	1.26×10^7	TNTC	15	0	7.100

[a]TNTC indicates Too Numerous To Count
[b] 0 indicates <10 cfu/ml

Table 19. Effect of elevated temperature on the survival of the *Pseudomonas* species in tryptic soy broth (Replicate 2).

		Number of Surviving Cells (cfu/ml)			
Temperature (˚C)	Initial Time	1 min	5 min	10 min	Log reduction @ 10 min
60	1.95×10^8	TNTC[a]	TNTC	TNTC	N/A
65	1.95×10^8	TNTC	0[b]	0	8.290
70	1.95×10^8	TNTC	0	0	8.290

[a]TNTC indicates Too Numerous To Count
[b] 0 indicates <10 cfu/ml

Temperature versus Exposure Time Using Saline:

The effect of elevated temperatures and exposure time was also assessed at 60˚C, 65˚C, and 70˚C using saline. The results indicate that exposure for 10 minutes at 70˚C results in at least a 7 log reduction in number for the environmental isolates when tested in saline. A replicate of *Pseudomonas aeruginosa 1* could not be completed due to viability of the isolate. (Tables 20 through 24)

Table 20. Effect of elevated temperature on the survival of *Pseudomonas aeruginosa 1* in saline (Replicate 1).

		Number of Surviving Cells (cfu/ml)		
Temperature (˚C)	Initial Time	5 min	10 min	Log reduction @ 10 min
60	7.2×10^7	TNTC[a]	3	7.380
65	7.2×10^7	3	13	6.743
70	7.2×10^7	0[b]	0	7.857

[a]TNTC indicates Too Numerous To Count
[b] 0 indicates <10 cfu/ml

Table 21. Effect of elevated temperature on the survival of *Pseudomonas aeruginosa 2* in saline (Replicate 1).

		Number of Surviving Cells (cfu/ml)		
Temperature (˚C)	Initial Time	5 min	10 min	Log reduction @ 10 min
60	9.5×10^7	15	5	7.279
65	9.5×10^7	0[a]	1	7.978
70	9.5×10^7	3	9	7.023

[a] 0 indicates <10 cfu/ml

Table 22. Effect of elevated temperature on the survival of *Pseudomonas aeruginosa 2* in saline (Replicate 2).

		Number of Surviving Cells (cfu/ml)		
Temperature (˚C)	Initial Time	5 min	10 min	Log reduction @ 10 min
60	5.4×10^7	0[a]	7	6.887
65	5.4×10^7	0	0	7.732
70	5.4×10^7	0	0	7.732

[a] 0 indicates <10 cfu/ml

Table 23. Effect of elevated temperature on the survival of the *Pseudomonas* species in saline (Replicate 1).

		Number of Surviving Cells (cfu/ml)		
Temperature (˚C)	Initial Time	5 min	10 min	Log reduction @ 10 min
60	2.50×10^8	TNTC[a]	TNTC	N/A
65	2.50×10^8	222	252	5.997
70	2.50×10^8	0[b]	3	7.921

[a]TNTC indicates Too Numerous To Count
[b] 0 indicates <10 cfu/ml

Table 24. Effect of elevated temperature on the survival of the *Pseudomonas* species in saline (Replicate 2).

		Number of Surviving Cells (cfu/ml)		
Temperature (˚C)	Initial Time	5 min	10 min	Log reduction @ 10 min
60	5.2×10^7	396	57	5.960
65	5.2×10^7	6	56	5.968
70	5.2×10^7	0[a]	0	7.716

[a] 0 indicates <10 cfu/ml

Conclusion/Discussion

In this study the environmental isolates recovered from the manufacturing environment of an alcohol-free mouthwash were investigated because of previous intrinsic microbial contamination. Due to the severity of the problems associated with intrinsic microbial contamination of this product and any pharmaceutical product, the source of the contamination warranted further scrutiny. The limitations of standard USP tests also require consideration when dealing with pharmaceutical products that can affect public health. USP standard tests are performed with designated organisms that are unlikely to be the microorganisms present in the manufacturing environment. When pharmaceutical products are tested, only the standard USP organisms are used. However, in the actual manufacturing environment the product comes into contact with many other species of microorganisms, such as the environmental isolates used in this study. In this study only three environmental isolates were used. However, several other isolates were found that could have been studied using the same tests to determine their susceptibility or resistance to the preservative system used.

The first objective of this study was to compare the United States Pharmacopeia (USP) Microbial Limits test using the standard USP organisms and the environmental isolates recovered at the manufacturing facility. The test procedure used for the USP Microbial Limits test was validated using the USP recommended organisms. In both replicates of the USP test, the alcohol-free mouthwash was certified as free from the designated organisms, and when present in low numbers, these organisms were recoverable by the standard procedure.

In the Microbial Limits test using the environmental isolates, the standard method was also validated, and when present in low numbers, the isolates were recoverable. The product was

also certified as free from *Pseudomonas aeruginosa isolates 1* and *2* and isolate 3, a *Pseudomonas* species.

The second objective was to evaluate the parabens preservative system used in the mouthwash. This was done by comparing the standard USP organisms with the isolates recovered from the manufacturing environment. The United States Pharmacopeia stipulates for a Preservative Effectiveness test that bacteria should have no less than a 1.0 log reduction by day fourteen of the test and no increase from the count at fourteen days. Yeasts should have no increase from the initial count at fourteen days and twenty-eight days (USP, 2006). In the standard USP test, *Pseudomonas aeruginosa* (ATCC #9027) exhibited resistant to the parabens preservative used in the alcohol-free mouthwash. In both replicates at least a two log reduction was observed for *P. aeruginosa* by week four of the test. However, all other isolates, *Staphylococcus aureus* (ATCC #6538), *Escherichia coli* (ATCC #8739), *Candida Albicans* (ATCC #10231) and *Aspergillus niger* (ATCC #16404) failed to grow after one week. Although under the USP stipulations the reduction in number of *P. aeruginosa* was sufficient, the resistance the microorganism exhibited is a concern. Parabens are known to be less effective against *Pseudomonas* species. This was indicated by the results of these tests (Hugo and Russel, 1983).

The results of the preservative effectiveness test when repeated with the environmental isolates indicated that *P. aeruginosa* again displayed a resistance to the preservative system used. *P. aeruginosa* isolates 1 and 2 exhibited at least a two log reduction by week four of the test. However, Isolate 3, a *Pseudomonas species* failed to grow by week one of the test. Under the manufacturing conditions, repeated exposure of the *Pseudomonas aeruginosa* isolates and *Pseudomonas species* to the mouthwash could lead to increased resistance over time.

The third objective of this study was to evaluate the survival of the environmental isolates when exposed to temperature between 60˚C and 70˚C. These tests were done in order to improve the manufacturer's ability to control further intrinsic microbial contamination. Controlling microbial contamination with elevated temperature rather than chemical agents is better for the manufacturer. When such agents are used, the manufacturer must demonstrate that no chemical residue is remaining in the mixing kettles before the actual product is produced. If temperature can be used with the same result as chemical agents, it would be more beneficial for the production of these products. These tests were also conducted in both tryptic soy broth (TSB) and saline to compare the effect of each on the isolates when exposed to the elevated temperatures. Results indicate that exposure for 10 minutes at 70˚C results in at least a seven log reduction in number for the environmental isolates when exposed in the presence of TSB. When exposed for ten minutes in TSB at 65˚C at least a seven log reduction from initial densities was observed. Results from exposure at 60˚C for ten minutes in TSB were variable and actual microbial counts, colony forming units, were not determined due to high survival rate. At least a five log reduction was observed for all three environmental isolates in at least one replicate of the test.

When the tests were repeated at 70˚C for ten minutes in the presence of saline, at least a seven log reduction was observed for *Pseudomonas aeruginosa 1, Pseudomonas aeruginosa 2*, and the *Pseudomonas species*. When exposed for ten minutes at 65˚C, at least a six log reduction in number was observed for the environmental isolates. Exposure at 60˚C for ten minutes also resulted in at least a five log reduction in number from the initial density levels.

The manufacturers of this mouthwash product and other similar pharmaceutical products that utilize weak preservative systems should routinely examine their manufacturing facilities to

help prevent the intrinsic contamination of their products. In the case of the alcohol-free mouthwash used in this study, sanitization procedures should be reviewed and at least 70˚C water should be used to rinse the mixing kettles after formulation of the product. Rinsing of the tanks should also be done for at least ten minutes. However a time period longer than this would result in a greater kill off of microorganisms. The garden hoses used should regularly be replaced, and water should be allowed to drain after each use.

References

Anderson, Roger L. et al. 1985. Factors Associated with *Pseudomonas pickettii* Intrinsic Contamination of Commercial Respiratory Therapy Solutions Marketed as Sterile. *Applied and Environmental Microbiology*. 50:1343-1348.

Anderson, Roger L., et al. 1983. Investigations into the Survival of *Pseudomonas aeruginosa* in Poloxamer-Iodine. *Applied and Environmental Microbiology*. 47: 757-762.

Anderson, Roger L., et al. 1990. Prolonged Survival of *Pseudomonas cepacia* in Commercially Manufactured Povidone-Iodine. *Applied and Environmental Microbiology*. 56:3598-3600.

Berkelman, Ruth L., et al. 1982, Increased Bactericidal Activity of Dilute Preparations of Povidone-Iodine Solutions. *Journal of Clinical Microbiology*. 15:635-639.

Berkelman, Ruth L. et al. 1983. Intrinsic Bacterial Contamination of a Commercial Iodophor Solution: Investigation of the Implicated Manufacturing Plant. *Applied and Environmental Microbiology*. 47:752-756.

Centers for Disease Control and Prevention. 1982. Epidemiologic Notes and Reports *Pseudomonas aeruginosa* Peritonitis Attributed to a Contaminated Iodophor Solution - - Georgia. Morbidity and Mortality Weekly. 32(15):197-198.

Centers for Disease Control and Prevention. 1989. Contaminated Povidone-Iodine Solution - - Texas. Morbidity and Mortality Weekly. 38(8):133-134.

Centers for Disease Control and Prevention. 1998. Nosocomial *Burkholderia cepacia* Infection and Colonization Associated with Intrinsically Contaminated Mouthwash - - Arizona 199. Morbidity and Mortality Weekly. 47(43):926-928.

Centers for Disease Control and Prevention. 2004. Nosocomial *Burkholderia cepacia* Infection Associated with Exposure to Sublingual Probes - - - Texas. Morbidity and Mortality Weekly. 53(34):796.

Centers for Disease Control and Prevention. 2005. Nosocomial Burkholderia cepacia pneumonia associated with contaminated alcohol-free mouthwash. Health Alert Network.

Craven, D. E. et al. 1981. Pseudobacteremia caused by povidone-iodine solution contaminated with *Pseudomonas cepacia*. *New England Journal of Medicine.* 305:621-623

Henderson, DK, et al. 1988. Indolent epidemic of *Pseudomonas cepacia* bacteremia and pseudobacteremia in an intensive care unit traced to a contaminated blood gas analyzer. *American Journal of Medicine.* 84(1):75-81.

Hugo, W B, A D Russel, *Pharmaceutical Microbiology* 3 Ed. Blackwell Scientific Pulications. Oxford. 1983.

Mackel, Donald C., et al. 1975. Nationwide Epidemic of Septicemia Caused by Contaminated Intravenous Products: Mechanisms of Intrinsic Contamination. *Journal of Clinical Microbiology*. 2:486-497.

McNeil, M.M., et al. 1985. Nosocomial *Pseudomonas pickettii* Colonization Associated with a Contaminated Respiratory Therapy Solution in a Special Care Nursery. *Journal of Clinical Microbiology*. 22:903-907.

Nosocomial *Burkholderia cepacia* Infection and Colonization Associated with Intrinsically Contaminated Mouthwash – Arizona, 1998. 1999. *The Journal of the American Medical Association*. 281:318.

Ralstonia pickettii Colonization Associated With Intrinsically Contaminated Saline Solution – Los Angeles, California, 1998. 1998. *The Journal of the American Medical Association*. 279:1859.

Tokars, Jerome I., et al. 1990. *Mycobacterium gordonae* Pseudoinfection Associated with a Contaminated Antimicrobial Solution. *Journal of Clinical Microbiology*. 28:2765-2769.

The United States Pharmacopoeia (USP 30/NF 25), 2006. vol:83-88

The United States Pharmacopoeia (USP 30/NF 25). 2006. vol:2499-2500.