SPECTRUM
ANTIMICROBIALS

March 5, 2023

Spectrum Antimicrobials has developed the first broad spectrum sporicidal, antibacterial, antiviral chemistries that they are still safe to be nebulized in humans. Through my past various companies, I was actively developing new hypochlorous acid (HOCl) based solutions for medical purposes over the last 28 years and have overseen more than $100M in research and development funds in this space – the result of which are the current breakthrough chemistries – Spectricept which is developed by our company today.

Our various product chemistries have received recognition, clearance and/or approval by the Food and Drug Administration (Spectricept Skin and Wound Cleanser) as well as by the National Health System (NHS), in the United Kingdom (Spectricept hand santizer). The NHS has issued our UK commercial partner a 3-year tender to purchase Spectricept – the only approved sporicidal hand sanitizer in the world. We believe our various chemistries have many applications within the Department of Defense which may include keeping personnel safe against biological hazards regardless of type or species of the pathogen.

Key advantages of our product chemistries are:

- Fast Acting – our product chemistries utilizes HOCl, which has detectible biologic activity in as little as 100s of a millisecond against bacteria as peer reviewed and published in medial journals. We have shown complete kill against C. Diff spores in as short as 2 minutes of exposure time; unlike other product chemistries which require exposures of up to 24-hours for safe eradication of possible pathogens.
- Resistant Microbes – our product's active ingredient a) eradicates antibiotic resistant viruses and bacteria and, b) works in a way that does not promote the emergence of new mutations.
- Safety – we believe our product safety is unparalleled as documented in our separate submissions to both agencies the FDA and the UK NHS.
- Patents – our chemistries are unique, novel and protected with composition of matter patents – 11 issued and 74 pending.
- Non-Alcohol Attributes - unlike alcohol-based chemistries, our product formulations are non-flammable, sourced from alternative origins unrelated to petroleum pricing and sources constraints, and most importantly, they resist freezing temperatures, which makes other product types and chemistries non-usable.

Our advances in chemistries have led us to create some of the most novel anti-infective formulations, which allow for better control, prevention and treatment of infectious diseases that remain safe and lower economic risks to our society.

I have attached herein an information package for your initial review and consideration. I hope we can be merited a brief interview in order to present our technology to the U.S. government as the only patented solutions in the world.

A. FDA clearance letter for Spectricept Skin and Wound Cleanser
 - Reference A
B. Letter by Johns Hopkins School of Medicine
 - Reference B
C. Serum Resistant Hypochlorous Acid Memorandum
 - Reference C (includes references 1-9)

Respectfully,

Hoji Alimi - CEO

Reference A.

FDA clearance – Spectricept Skin and Wound Cleanser

January 13, 2023

Spectrum Antimicrobials, Inc. (Subsidiary of Collidion, Inc.)
℅ Dana Dunn
Principal
Dunn Regulatory Associates, LLC
2709 Silkwood Court
Oakton, Virginia 22124

Re: K213514
Trade/Device Name: Spectricept Skin and Wound Cleanser
Regulatory Class: Not classified
Product Code: FRO
Dated: October 29, 2021
Received: November 2, 2021

Dear Dana Dunn:

We have reviewed your Section 510(k) premarket notification of intent to market the device referenced above and have determined the device is substantially equivalent (for the indications for use stated in the enclosure) to legally marketed predicate devices marketed in interstate commerce prior to May 28, 1976, the enactment date of the Medical Device Amendments, or to devices that have been reclassified in accordance with the provisions of the Federal Food, Drug, and Cosmetic Act (Act) that do not require approval of a premarket approval application (PMA). You may, therefore, market the device, subject to the general controls provisions of the Act. Although this letter refers to your product as a device, please be aware that some cleared products may instead be combination products. The 510(k) Premarket Notification Database located at https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpmn/pmn.cfm identifies combination product submissions. The general controls provisions of the Act include requirements for annual registration, listing of devices, good manufacturing practice, labeling, and prohibitions against misbranding and adulteration. Please note: CDRH does not evaluate information related to contract liability warranties. We remind you, however, that device labeling must be truthful and not misleading.

If your device is classified (see above) into either class II (Special Controls) or class III (PMA), it may be subject to additional controls. Existing major regulations affecting your device can be found in the Code of Federal Regulations, Title 21, Parts 800 to 898. In addition, FDA may publish further announcements concerning your device in the Federal Register.

Please be advised that FDA's issuance of a substantial equivalence determination does not mean that FDA has made a determination that your device complies with other requirements of the Act or any Federal

statutes and regulations administered by other Federal agencies. You must comply with all the Act's requirements, including, but not limited to: registration and listing (21 CFR Part 807); labeling (21 CFR Part 801); medical device reporting (reporting of medical device-related adverse events) (21 CFR 803) for devices or postmarketing safety reporting (21 CFR 4, Subpart B) for combination products (see https://www.fda.gov/combination-products/guidance-regulatory-information/postmarketing-safety-reporting-combination-products); good manufacturing practice requirements as set forth in the quality systems (QS) regulation (21 CFR Part 820) for devices or current good manufacturing practices (21 CFR 4, Subpart A) for combination products; and, if applicable, the electronic product radiation control provisions (Sections 531-542 of the Act); 21 CFR 1000-1050.

Also, please note the regulation entitled, "Misbranding by reference to premarket notification" (21 CFR Part 807.97). For questions regarding the reporting of adverse events under the MDR regulation (21 CFR Part 803), please go to https://www.fda.gov/medical-devices/medical-device-safety/medical-device-reporting-mdr-how-report-medical-device-problems.

For comprehensive regulatory information about medical devices and radiation-emitting products, including information about labeling regulations, please see Device Advice (https://www.fda.gov/medical-devices/device-advice-comprehensive-regulatory-assistance) and CDRH Learn (https://www.fda.gov/training-and-continuing-education/cdrh-learn). Additionally, you may contact the Division of Industry and Consumer Education (DICE) to ask a question about a specific regulatory topic. See the DICE website (https://www.fda.gov/medical-devices/device-advice-comprehensive-regulatory-assistance/contact-us-division-industry-and-consumer-education-dice) for more information or contact DICE by email (DICE@fda.hhs.gov) or phone (1-800-638-2041 or 301-796-7100).

Sincerely,

Julie A. Morabito, Ph.D.
Assistant Director
DHT4B: Division of Infection Control
and Plastic Surgery Devices
OHT4: Office of Surgical
and Infection Control Devices
Office of Product Evaluation and Quality
Center for Devices and Radiological Health

Enclosure

Reference B.

Johns Hopkins School of Medicine, Letter

Anesthesiology & Critical Care Medicine
Jerry Stonemetz, Medical Director, Perioperative Services
Medical Director, Center for Perioperative Optimization
1800 Orleans St; Sheik Zahed Tower 4163
Baltimore, MD 21287-0712
410-955-2521
jstonemetz@jhmi.edu

August 31, 2020

Office of Senator John Kennedy
101 La Rue France suite 503
Lafayette, LA 70508

Hypochlorous Acid as an Effective Inhaled Antiviral Treatment

According to our Epidemiologists here at Johns Hopkins, "We are still in the first wave" of the pandemic. The global COVID-19 pandemic has engulfed the citizens of all nations around the globe resulting in millions of deaths while impacting both global commerce and increasing national security concerns. We believe that advanced and science-based solutions must be deployed in order to help curtail the pandemic wave and ultimately restoring modest order for the global economy to progress forward.

Over the last few decades, researchers at Spectrum Antimicrobials in the United States have captured the power of the human immune system to manufacture the first stable drug formulation of HOCl to combat viral and bacterial infections including those caused by COVID-19. The same drug active has been now commercialized as a hand sanitizer and disinfectant in The United Kingdom and other countries with plans to initiate human clinical trials to investigate the safety and efficacy of Spectricept 115HP drug using a novel chemistry to treat COVID patients.

HOCl, a weak acid of chlorine is produced by human immune systems, where it is produced by our defensive cells (phagocytes) to attack foreign bodies at a cellular level. Once produced, it is extremely effective at killing bacteria, spores and viruses. It rapidly degrades to harmless saline solution. HOCl has been recognized as a potent antimicrobial for decades, however, the challenge was the ability to introduce complementary known additives to assist the low concentrations of this drug to yield a stable and effective formulation to treat patients. Spectricept 115HP contains 115 ug/L HOCl as well as stabilized oxygen and hydrogen peroxide at safe concentrations of less than 75mg/L. Over the last 20 years, HOCl has been well researched, published and also cleared by FDA in different therapeutic areas including reduction of topical inflammation and topical pain, management of chronic wounds as well as disinfection of food and food preparation areas in the consumer market. However, none of the above-mentioned products have been able to provide the required stability, safety and efficacy as a drug to meet FDA's rigorous standards to date. Spectrum Antimicrobials unique and patented 115HP is designed with specific additive chemistry to provide the required antiviral and antibacterial therapeutics while maintaining tissue compatibility and safety.

We have been investigating this chemistry for possible treatment of patients suffering from COVID-19 symptoms. The company is currently collaborating with several key physicians in Austria, Germany and Italy to initiate the first human clinical trial to evaluate the safety and efficacy of Spectricept 115HP drug. Initial results have been profoundly promising with two COVID positive patients suffering from high fever and shortness of breath fully recovering within 48-72 hours post initial treatment. A formal controlled trail in expected to be underway within 30 days. We strongly believe that this newly developed chemistry of novel Hypochlorous acid with stable oxygen and hydrogen peroxide constituents will demonstrate to be an ideal

candidate for treatment of COVID positive patients. The company has been successful in launching the same drug active component:

- As a hand sanitizer in United Kingdom and expects to open other parts of Europe and international markets.
- Plans to file for FDA 510K clearance for the use of this active in management of chronic and acute wounds in Q3 2020.
- Lastly, the company is now launching a special chemistry of this drug active for use in ventilators and CPAPs for prevention of growth of viruses and bacteria in The U.S.

In its stable form, a key feature of HOCl which has been well studied and published is its ability to pose non-selective antiviral and antibacterial effect against a broad range of viruses and bacteria, without inducing further microbial resistance. Studies to date show HOCl does not rely on a single mode of action to block or eradicate viruses and bacteria and further confirms it does not induce resistance amongst target viruses and bacteria.

Covid-19 virus (SARS CoV2) has an envelope or outer coating that is composed of a lipid layer (fatty substance). This is the virus's weak point since breaching the envelope results in complete loss of infectivity ("kills" the virus), and this can be accomplished by appropriate disinfectant chemistry. Spectricept has been independently tested against a range of bacteria and other microorganisms that are known to be difficult to "kill". Spectricept has demonstrated to be highly effective against corona virus when tested at a third party laboratory in the U.S. Spectricept is the only HOCl based solution to date capable of demonstrating the required shelf-life and stability while also capable of providing consistent antimicrobial activity in presence of soil and serum load.

Shortly after Covid-19 began manifesting infections in this country, we began getting feedback from physicians in Wuhan, China. One of the very concerning aspects of this virus was the very high susceptibility of head and neck surgeons to getting infected. It has been shown that this virus remains in extremely high concentrations within the nasal passages, and is likely to reside there for a few days before systemic infections appear. Based on this realization, the physicians on the Advisory Board at Spectrum proposed that HOCl could potentially be used as an inhaled treatment to reduce viral load, and may potentially reduce or eliminate the progression of disease. Their findings were also based on previous studies that demonstrated that low concentrations of HOCl are effective and safe as a nasal irrigation against nasal infections including viruses. Consequently we requested that Spectrum create a specific formulation of HOCl that would be the safest platform for inhalation and that product is known internally to the company as Spectricept 115HP.

Higher concentrations of Spectricept 115HP has been subjected to full biocompatibility testing in compliance with the ISO 10993 series of standards. Spectricept has successfully passed the following safety studies conducted at a certified third-party laboratory.

- *Cytotoxicity; Cells did not show any toxicity*
- *Hemolysis; blood cells did not show any toxicity*
- *Skin irritation; Exposure to animal skin showed no irritation*
- *Skin sensitization; Repeated exposure to animal skin showed no signs of sensitization*
- *Systemic injection; direct injection into animal showed no sign of systemic toxicity*
- *Pyrogenicity; direct injection into blood stream of animal showed no increase in body temperature*

- *AMES test: the results indicate the drug formulation is non-mutagenic.*

In conclusion, based on the scientific information provided by Spectrum, the Spectricept solution has shown to be a highly effective (antiviral and antimicrobial) drug and it has been shown to be biocompatible at the concentration levels referenced herein. As such, I would recommend that Spectricept 115HP should be considered and evaluated by authorities as an ideal potential candidate for use as a first line of defense against Covid-19 as an inhaled therapy. We are seeking your assistance to find possible collaborators within the VA hospital systems to possibly accelerate the first in human clinical trials in The U.S. for Spectricept 115HP. We are continuing to complete the requirements for an Investigation New Drug (IND) with the FDA but it will not be completed until 2021. It is my understanding that the VA hospital has the authority to pursue a human investigational study independent of the FDA if the drug merits such clinical investigation.

Based on my years of clinical experience, I am highly confident about Spectricept 115HP and its effectiveness in altering the trajectory of the pandemic through the means of preventing the spread of the disease as well as treating those who have been already infected. I feel this might represent the only true opportunity to keep this virus at bay. I am convinced that we are simply seeing the tip of the iceberg as to potential uses of this drug. I am more than happy to provide additional information if necessary.

Jerry Stonemetz, MD

Chief Medical Officer, Spectrum Antimicrobials, USA
Medical Director, Perioperative Services
Anesthesia & Critical Care Medicine
Johns Hopkins University
jstonemetz@jhmi.edu
410 955-2521

Reference C.

Serum Resistant Hypochlorous Acid Memorandum

(all further references (#1 - #9) relate to this memorandum)

Spectrum Antimicrobials, Inc.

Serum Resistant Hypochlorous Acid

Research and Development Memorandum

Prepared by:

Hoji Alimi – CEO and Head of Product Development

February 22, 2023

INTRODUCTION

This memorandum is intended to provide general references to certain applicable FDA and EPA regulations for the sole purpose of clarifying our rationale for the use of 5% serum, which is used in certain microbiological tests by Spectrum Antimicrobials. This document is not intended to serve as a complete guide to FDA and EPA regulations for testing, approval and regulation of antimicrobial products.

REGULATION PERTAINING TO ANTIMICROBIAL PRODUCTS

Surface disinfectants sold for use in both consumer and hospital markets are regulated by the U.S. Environmental Protection Agency (EPA). Medical devices and drugs are regulated by the U.S. Food and Drug Administration (FDA). However, registration of antimicrobial products/liquid chemical germicides with medical device claims are regulated under the Memorandum of Understanding between EPA and FDA (Reference 2 and 5) with both agencies maintaining and sharing oversight. Since the EPA registration requirements for general purpose disinfectants parallel the requirements necessary to receive marketing clearance for general purpose disinfectants under section 510(k) of the FD&C Act, then fulfillment of EPA's registration requirements fulfills FDA's section 510(k) requirements for those products (Reference 2 – Page 4 – last paragraph). These requirements may include the use of 5% serum when applicable pertaining to certain claims (Reference 5).

Authority for granting regulatory approval(s) to control, prevent and treat viral, bacterial and fungal infections using drug actives, topical and or localized solutions (I.e. eye, pulmonary, etc) on animals and humans remain under the sole jurisdiction of the FDA. The FDA determines the necessary studies to satisfy such regulatory application(s) (i.e. PMA, 510K, DeNovo, New Drug Application (NDA), Abbreviated New Drug Application (ANDA) or a biologic related application, etc.). These studies may involve one or both in vivo and or in vitro tests, that are deemed necessary to evaluate the efficacy and safety of a product prior to consideration for filing a regulatory submission, entry into human clinical trials or final approval. Medical companies do have the flexibility to design such tests based on their own understanding of the actives and scientific rationale to generate the required safety and efficacy information, and the FDA may provide feedback on such test designs when applicable. For example, the planned microbiological tests of such antiviral and antibacterial drug candidates may involve the use of specific human cell lines, small rodent studies, etc.

In contrast, regulations pertaining to general germicidal solutions designed to eradicate or halt (bacteriostatic) the growth of bacteria, spores, fungi and viruses within or on a medical device or on other inanimate surfaces and depending on the claims made by the manufacturer may be regulated by either or both FDA and EPA. The joint regulatory oversight is conducted by FDA and EPA in accordance with the Memorandum of Understanding mentioned above (Reference 2). For example, disinfection of endoscopes requires testing of germicidal products per EPA regulations using AOAC standards (Reference 4, 7) which are then used to submit to the FDA for further review and approval. The testing parameters under AOAC guidelines require strict use of pathogen types, exposure times, carriers, number of batches and test methods which cannot be modified and or altered for any reason including if the scientific rationale may exist (reference 6). This has been a significant barrier to market entry for all stable HOCl-based products to successfully achieve full sterilization (6-log reduction) in presence of 5% serum (reference 5) with concentrations below 500ppm to avoid toxicity and corrosion. Higher

concentrations of HOCl poses toxicity, irritation and shortened shelf life and stability. Therefore, a Serum Resistant HOCl at low concentration which can pas such testing in presence of 5% serum will expand its commercial use as a blockbuster chemistry on the global scale;

- fully replacing existing germicidal(s) and forms of generic HOCl cleared to date by FDA and EPA.
- Unlike other stable forms of HOCl, the unique chemistry of Spectricept™ withstands freezing temperatures during transit and distribution whereas other forms of generic stable HOCl are instantly deactivated.
- Spectricept composition of matter chemistry patent is a barrier to market entry for competitors.
- Therefore, Spectrum has a greater opportunity with its partners to expand label indications and commercial reach globally.

Stable HOCl products have historically failed to achieve composition of matter chemistry patent and/or failed to pass the 5% serum load test at reasonably safe concentration as referenced herein. Therefore, we decided to use the 5% serum load as a benchmark to compare the viability of our patent protected HOCl formulations against other competing products. This benchmark allows us to determine the product microbial activity during the serum load recommended by the applicable regulations.

OUR PRODUCTS

Spectricept™ Wound and Skin Cleanser is a Serum Resistant Hypochlorous Acid (HOCl) and represents a novel formulation chemistry of HOCl with the following key attributes:

- Composition of matter patent protection

- In vitro microbiology testing demonstrating antimicrobial effectiveness in laboratory testing against wide range of pathogens including antibiotic resistant bacteria in the presence of 5% serum (Reference 8)

- Although recommended temperatures for storage and handling of Spectricept™ is at ambient temperature. (20C-25C), the product has demonstrated the ability to surpass other HOCl solutions by withstanding freezing conditions thus reducing distribution related challenges and added cost of using temperature controlled trucks.

We filed a 510(k) application for the use of Spectricept™ as a wound and skin cleanser in November 2021. Spectricept™ Wound and Skin Cleanser received FDA 510(k) clearance for use in both acute and chronic wounds in January 2023. To support this filing, we completed separate animal studies to demonstrate the safety of Spectricept™ product in various tests and animal models.

RATIONALE FOR APPROVAL OF SPECTRICEPT™ BY THE FDA

As noted above, Spectricept™ Wound and Skin Cleanser is cleared as a 510K medical device. We provided the following statements and scientific rationale to the FDA explaining the urgent need for inclusion and use of a Serum Resistant HOCl in Spectricept™. FDA used the following information to evaluate the medical need for the use of Spectricept™ as a criteria to grant us our final 510K clearance.

- Infection is considered a serious clinical concern which may prevent or delay the process of wound healing, cause sepsis and even death, if not treated. Furthermore, it is understood that infected wounds do not heal, and the progression of infection may lead to further costly clinical complications.
- Prevention is a recognized critical step in lowering hospital acquired infections (HAIs) and its associated cost to the overall healthcare system. According to CDC "HAIs in U.S. hospitals have direct medical costs of at least $28.4 billion each year" (reference 1). The use of an effective wound care solution containing a Serum Resistant HOCl can drastically contribute to the overall reduction of incidences of HAI's.
- The emergence of antibiotic resistant bacteria (i.e., *Methicillin-resistant staphylococcus aureus (MRSA)*, *Vancomycin-resistant Enterococcus (VRE)*, amongst others) have significantly lowered the effectiveness of existing antibiotics routinely administered to treat and prevent such infections.
- Wound care management and dressing changes are routinely performed in non-sterile environments. Therefore, the use of an efficacious solution to prevent entry and harboring of infectious diseases into wound wash closure systems (Reference 7) as evident by peer reviewed published sources which help document spores of Clostridioides difficile to become airborne. This can lead to contamination of wound closure systems which can then be transferred from one wound care session to the next infecting new patients (reference 9, 7).
- Infection may lead to comorbidities, which also may further decrease patient safety.

In recognition of the need for alternative products to improve the overall health care and to reduce the burden of infections on the healthcare system, the FDA has cleared several hypochlorous acid-based solutions, since the early 2000s, for use in the debridement and cleaning of acute and chronic wounds. These products are packaged using industry standard packaging with a spray nozzle which operates to spray the solution uniformly onto the wound bed. These bottle closure systems mechanically suction non-sterile air containing potential harmful infections diseases to replace any volume of product which exits the closure system. Introduction of contaminated air into sterile bottles containing wound solutions with secondary application of such solution onto open wounds of other patients may pose concern for the spread of nosocomial infections (Reference 9).

According to peer reviewed and published data, the underlying cause for the spread of nosocomial infections is not limited to potential exposure to = surfaces, tools and compromised aseptic processes and procedures. Therefore, opportunistic infectious diseases transmittable through air can compromise open wounds, surgical site incisions, etc. in hospitals.

Under applicable FDA regulations, HOCl shall be responsible to maintain the integrity of the wound care solution when used in non-stile environments where infectious diseases may be present. Wound care management and irrigation of surgical sites during initial use, and secondary storage of such solutions for later use and the follow on re-use of these same solutions may expose patients unnecessarily to second degree contaminations and an overall higher rate of nosocomial infections. Generic stable HOCl concentrations naturally degrade over time. Therefore, lower than expected HOCl concentrations that are not serum resistant may become more susceptible to harboring organic and bacterial loads during aging process, if not well studied and controlled.

Therefore, incorporation of a serum resistant HOCl - Spectricept™ ;

- Provides an unparalleled combination of safety and efficacy of more than one million fold over existing generic stable HOCl products which have been previously cleared by FDA as 510K products (reference 3).
- Demonstrates resistance to cold temperatures during transit ultimately responsible for interruptions for steady distribution of HOCl based products during winter in Mid West and East coast regions of the United States. Stable HOCl solutions are immediately deactivated by the process of freezing. Spectricept™ remains highly effective against pathogens even after freezing.
- Unlike antibiotics, Spectricept™ does not allow for emergence of new resistant pathogens.

Therefore, Soectricept™ is a high valued product which is designed to lower the rate of noscomial infections in hospital and clinical settings.

According to CDC "HAIs in U.S. hospitals have direct medical costs of at least $28.4 billion each year" (reference 1). The use of an effective wound care solution containing serum Resistant HOCl can drastically contribute to the overall reduction of incidences of HAI's.

REFERENCES

1- CDC Reference – cost of HAIs
https://www.cdc.gov/policy/polaris/healthtopics/hai/index.html

2- Memorandum of Understanding Between the Food and Drug Administration Public Health Service, Department of Health and Human Services and Department of Environmental Protection Services - https://www.epa.gov/sites/default/files/2014-09/documents/pr94-4-mou-1.pdf

3- FDA Submission - SUMMARY OF EFFICACY TESTING OF SPECTRICEPT PRODUCT VERSUS HOCI

4- US Environmental Protection Agency Office of Pesticide Programs - UDM Performance Standard Revision Document

5- Product Performance Test Guidelines - OCSPP 810.2200: Disinfectants for Use on Hard Surfaces—Efficacy Data Recommendations

6- US EPA – Office of Pesticide Programs. Standard Operating Procedure for the AOAC Sporicidal activity of disinfectant tests

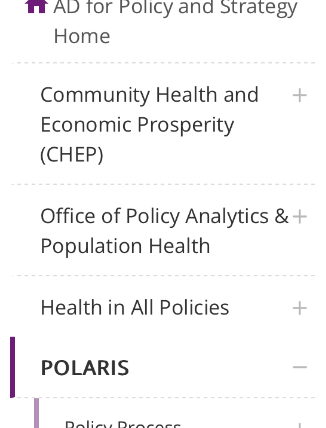
7- FDA Submission – Bottle Closure System Leak test report

8- In dependent laboratory - Time Kill Study Per USP Guidelines

9- Peer reviewed published paper – A comparison of air sampling methods for clostridium difficile edospore aerosol

Reference #1

CDC Reference – cost of HAIs



AD for Policy and Strategy Home
Community Health and Economic Prosperity (CHEP) +
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Health in All Policies +
POLARIS –
Policy Process +
Economic Evaluation +
Thinking in Systems +
Rural Health +
Policy Resources and Training +
Health Topics –
Adverse Childhood Experiences (ACEs)

Health Topics – Healthcare-associated Infections (HAI)

Print

Overview

HAIs are infections resulting from complications of healthcare. They are linked with high morbidity and mortality. On any given day, 1 in 31 hospital patients has an HAI (an infection while being treated in a medical facility). Additional infections occur in other healthcare settings. Many HAIs are caused by the most serious antibiotic-resistant bacteria and can lead to sepsis or death.

Economic Burden

HAIs in U.S. hospitals have direct medical costs of at least $28.4 billion each year. They also account for an

Reference #2

Memorandum of Understanding Between the Food and Drug Administration Public Health Service, Department of Health and Human Services and Department of Environmental Protection Services -

Memorandum of Understanding
Between
The Food and Drug Administration, Public Health Service,
Department of Health and Human Services
and
The Environmental Protection Agency

Notice Regarding Matters of Mutual Responsibility - Regulation of Liquid Chemical Germicides Intended for Use on Medical Devices

I. PURPOSE

This Memorandum of Understanding (MOU) between the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) clarifies jurisdiction between the two agencies in the regulation of certain liquid chemical germicides. These liquid chemical germicides are devices under the Federal Food, Drug, and Cosmetic Act (FD&C Act) and pesticides under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). This MOU also embodies the agreement of the two agencies to undertake certain rulemakings in order to eliminate duplicative regulation of certain types of liquid chemical germicides. This MOU includes the agencies' interim agreement to simplify and coordinate their regulatory and enforcement activities in shared areas of jurisdiction affecting these types of products pending the conclusion of these rulemakings.

II. STATUTORY AUTHORITIES

A. FDA Authorities

The FD&C Act grants FDA authority to regulate devices as defined in 21 U.S.C. 321(h). Under section 321(h), the term "device" includes an instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent, or other similar or related article, including any component, part, or accessory that is intended to cure, mitigate, treat, or prevent disease in man, or is intended to affect the structure or any function of the body of man. Liquid chemical germicides intended for use in conjunction with a variety of articles that fit within the statutory definition of "device," such as operating instruments, medical examining tables, hospital scales, and other hospital equipment, also fall within the definition of "device" because they are considered accessories to these devices.

Unless liquid chemical germicides used in conjunction with devices were commercially distributed prior to May 28, 1976,1 manufacturers of these products, under 21 U.S.C. 360(k) [section 510(k) of the FD&C Act] are required to submit a premarket notification to FDA before they market their products. Before these products can be legally marketed, FDA must grant marketing clearance by (1) issuance of an order in response to a section 510(k) submission which exempts the device from the FD&C Act's

premarket approval requirements, or (2) approval of a premarket approval application. In granting marketing clearance by issuance of a section 510(k) order exempting a liquid chemical germicide from premarket approval, FDA must find that the device is "substantially equivalent," as the term is defined in 21 U.S.C. 360c(i)(1)(A), to a predicate device that does not require premarket approval. Section 513 of the FD&C Act authorizes FDA to exempt products from premarket notification requirements for which there is a reasonable assurance of safety and effectiveness. At present, no chemical germicides that are used with devices have been exempted from premarket notification requirements.

In regulating liquid chemical germicides used with devices, FDA is exercising its responsiblilities under the FD&C Act for ensuring that devices are safe and effective for their intended uses. The FD&C Act provides enforcement authority to FDA to pursue regulatory actions, including seizure, injunction, prosecution, and civil penalties.

B. EPA Authorities

Liquid chemical germicides, including those regulated as devices, are also under the authority of the EPA under FIFRA. Before a pesticide product may be lawfully sold or distributed in commerce, the product must be registered by EPA pursuant to FIFRA section 3, or otherwise exempted from the requirements of FIFRA. A registration is a license allowing a pesticide product to be sold and distributed for specified uses in accordance with specified use instructions, precautions, and other terms and conditions. Liquid chemical sterilants are included among the various types of antimicrobial products that are currently subject to FIFRA.

1/ Devices marketed prior to May 28, 1976 are grandfathered from the FD&C Act's premarket notification requirements. Neither FDA nor EPA are aware of any currently marketed products that are exempt under this grandfather provision. Should any exist, they are not covered by this Memorandum of Understanding.

A pesticide product may be registered or remain registered only if it meets the statutory standard for registration. Among other things, a pesticide must perform its intended pesticidal function without causing "unreasonable adverse effects on the environment" (FIFRA section 3(c)(5)). "Unreasonable adverse effects on the environment" is defined as "any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of [the] pesticide" (FIFRA section 2(bb)).

The burden of demonstrating that a pesticide product satisfies the statutory criteria for registration is at all times on the proponents of initial or continued registration. FIFRA section 6 provides EPA with various regulatory tools that the Administrator may use if it appears that the product no longer satisfies the statutory criteria for registration. If appropriate, EPA may require modifications to the terms and conditions of registration, such as deletion of particular uses or revisions to labeling, as an alternative to regulatory outcomes such as cancellation, suspension, or emergency suspension. FIFRA also provides enforcement authority to EPA to pursue actions, including issuance of stop sale, use, or removal orders when there is reason to believe a pesticide is in violation of FIFRA. Additionally, EPA has authority to seek the assessment of civil administrative penalties as well as institute seizure and criminal actions for violations of FIFRA.

FIFRA section 25(b) authorizes the Administrator to exempt pesticides from FIFRA through regulation if the Administrator determines that the pesticide is "adequately regulated by another Federal agency" or is "of a character which it is unnecessary to be subject to this Act in order to carry out the purposes of this Act."

III. REGULATORY RESPONSIBILITIES AND DEFINITIONS

For the purposes of this agreement, liquid chemical germicides that are used in conjunction with medical devices are divided into two product categories: (1) sterilants and (2) general purpose disinfectants. Sterilants, for purposes of this agreement, means those chemical germicides used to reprocess reusable critical and semicritical devices2. Critical devices are devices that are introduced directly into the human body, either into or in contact with the bloodstream or normally sterile areas of the body. These critical devices must be sterile.

2/ This definition is consistent with the definition of these terms used by the Centers for Disease Control and Prevention (CDC). Block, S.S. 1991. Disinfection, Sterilizaton, and Preservation. 4th Edition. Philadelphia, Lea & Febiger.
Semicritical devices are those which contact intact mucous membranes but which do not ordinarily penetrate the blood barrier or otherwise enter normally sterile areas of the body. For these devices, sterilization is desirable but not mandatory. These devices must be subjected at least to a high level disinfection3 process using a sterilant, but for a shorter time than that required for sterilization.

The second category of liquid chemical germicides are general purpose disinfectants. General purpose disinfectants, for purposes of this agreement, means those chemical germicides used to reprocess noncritical devices and medical equipment surfaces4.

Noncritical devices and medical equipment surfaces must be subjected to intermediate or low level disinfection5.

FDA's priority is to confirm the efficacy and safety of sterilants used to reprocess critical and semicritical devices which pose the greatest risk of disease transmission. This includes assuring that they do not adversely affect device performance or pose a hazard to the patient/user. Historically, EPA has assessed the effective performance of all chemical germicides and addressed health and safety issues presented by their use.

The FD&C Act and FIFRA have overlapping regulatory schemes for liquid chemical germicides used on devices. The objective of this MOU is to minimize redundant regulation of these products by FDA and EPA while assuring that the safety and efficacy requirements of both statutes are met. This affects three areas: data requirements for obtaining approval, procedures for obtaining approval, and compliance.

3/ "High level disinfectant" and "high level disinfection" are terms of art used by the public health community. FDA recognizes "high level disinfectant" as a separate or subcategory of sterilants. EPA does not register "high level disinfectants" as separate antimicrobial pesticides, but instead may register uses of germicides that correspond with uses in FDA's "high level disinfection" category.

4/ This definition is consistent with the definition of the term used by CDC.

5/ "Low and intermediate level disinfectants" are terms of art used by the public health community. FDA recognizes "low and intermediate level disinfection" as subcategories of general purpose disinfectants. EPA does not register low level and intermediate level disinfectants, but has corresponding germicide classes.

In determining whether the FD&C Act's and FIFRA's statutory and regulatory requirements are met, EPA and FDA will utilize the data requirements and performance standards referenced in FDA's current Guidance on the Content and Format of Premarket Notification Submission for Liquid Chemical Germicides, FDA premarket notification regulations at 21 CFR Part 807, Subpart E, EPA data requirements regulations at 40 CFR Part 158, and EPA's Subdivision G, Product Performance Guidelines.

Since the EPA registration requirements for general purpose disinfectants parallel the requirements necessary to receive marketing clearance for general purpose disinfectants under section 510(k) of the FD&C Act, fulfillment of EPA's registration requirements fulfills FDA's section 510(k) requirements for those products.

The EPA efficacy data requirements for liquid chemical sterilants, including those with high level disinfectant uses, are fulfilled by FDA's section 510(k) requirements or premarket approval requirements. Therefore, premarket clearance by FDA fulfills certain EPA registration requirements for liquid chemical sterilants, insofar as efficacy and product performance are concerned. FDA premarket clearance does not satisfy EPA's chemistry, toxicology, and ecological effects requirements.

IV. AGREEMENT

The Administrator of the Environmental Protection Agency and the Commissioner of the Food and Drug Administration agree that until exemptions referred to in Section V occur, the following division of responsibility will govern the activities of the agencies in the regulation of liquid chemical germicides that are intended for use on devices:

A. Regulatory Responsibilities

1. FDA will be primarily responsible for the premarket review of safety and efficacy requirements for liquid chemical germicides that are sterilants6 intended for use on critical or semicritical devices. Examples of critical devices are laparoscopes, surgical instruments, heart-lung oxygenators, and transfer forceps. Examples of semicritical devices are gastrointestinal endoscopes, endotracheal tubes, cystoscopes, anesthesia breathing circuits, and vaginal specula. FDA will also be primarily responsible for premarket review of contact lens solutions.

6/ If a liquid chemical sterilant product has subordinate claims such as tuberculocidal or virucidal, these claims also will be regulated by FDA.

2. EPA will be primarily responsible for premarket review of liquid chemical germicides that are general purpose disinfectants7 intended for use on devices other than critical or semicritical devices. Examples of noncritical devices are wheel chairs, medical beds, stands, certain operating room surfaces, medical lamps, dental units, and stethoscopes.

3. FDA marketing clearance through the section 510(k) process or approval through the premarket approval process of sterilants will satisfy certain requirements for registration under FIFRA Section 3. Upon submission to EPA by the applicant of an order issued by FDA granting marketing clearance or approval for a liquid chemical germicide that is a sterilant, EPA will consider the efficacy data requirements for registration to be satisfied, and will promptly determine whether the other requirements for registration are satisfied.

4. EPA registration of liquid chemical germicides that are used as disinfectants for devices, except sterilants, will satisfy the criteria necessary to establish substantial equivalence as defined in 21 U.S.C. 360c(i)(1)(A). For this category of liquid chemical germicides, submission by the manufacturer to FDA of a copy of the EPA correspondence granting registration will satisfy FDA's requirement for a premarket notification under 21 U.S.C. 360(k). Upon receipt of this information from the manufacturer of a liquid chemical germicide in this category, FDA will issue an order finding the product substantially equivalent to a predicate device that does not require premarket approval. This order will allow the device to be legally marketed without an approved FDA premarket approval application.

7/ Procedures described in Paragraph 4 only apply to liquid chemical germicide products that do not contain any sterilant claims. If a liquid chemical germicide product contains both sterilant and general purpose disinfectant claims, registration will proceed according to the procedures described in Paragraph 3. If the registrant of a general purpose disinfectant product registered by EPA subsequently applies for registration of a sterilant claim, registration of that product must proceed under procedures described in Paragraph 3 and the existing EPA registration will become void upon FDA's clearance of the product.

5. As part of the EPA registration process, EPA will require registrants of liquid chemical germicides, other than sterilants that have received FDA premarketing clearance or approval, to put the following statement on their product labels:

"This product is not to be used on any surface or instrument that (1) is introduced directly into the human body, either into or in contact with the bloodstream or normally sterile areas of the body, or (2) contacts intact mucous membranes but which does not ordinarily penetrate the blood barrier or otherwise enter normally sterile areas of the body."

B. Compliance Responsibilities

1. FDA will be responsible for all sampling and all efficacy testing of liquid chemical sterilants intended for use on critical and semicritical devices and for instituting appropriate enforcement and/or regulatory action against any products that do not comply with the FD&C Act.

Upon request, EPA will provide FDA with copies of the latest accepted labeling and the name and location of the production site for each product FDA intends to sample.

To the extent allowed under 21 U.S.C. 331j, 21 U.S.C. 360(j)(c), 42 U.S.C. 263g(d), 42 U.S.C. 263i(e), and 21 C.F.R. Part 20, FDA will share all safety and efficacy test results, labeling changes, and upon EPA request, any other information obtained during FDA enforcement/regulatory actions relating to liquid chemical sterilants. EPA may use this information to determine whether the registrant has complied with FIFRA. On the basis of this information, EPA may determine that further regulatory action under FIFRA, including cancellation of the product's registration, is warranted.

2. EPA will be responsible for the sampling and efficacy testing of all general purpose chemical germicides that are intended for use on devices other than critical and semicritical devices, and for instituting appropriate enforcement and/or regulatory action against any such chemical germicide that does not comply with FIFRA. EPA will refer labels and other evidence concerning inefficacious liquid chemical germicides intended for use on medical devices other than critical or semicritical to FDA for complementary action under the FD&C Act.

3. Each agency will provide assistance upon request to support compliance activities and litigation by the other Agency in cases involving liquid chemical germicides that are intended for use on devices. Assistance will be requested in accordance with applicable procedures, statutory and regulatory requirements, including compliance with regulations of 21 CFR Part 20, through the liaison officers listed below. Assistance may include provision of sampling, inspection and audit data, expert witnesses, certified statements, and affidavits.

Each Agency may consult with the other at any time to determine if the initiation of regulatory and/or enforcement action against a liquid chemical germicide in lieu of or concurrently with the other agency's action is appropriate.

This Memorandum of Understanding has no effect on any pending investigations or enforcement or regulatory actions undertaken by EPA pursuant to FIFRA or by FDA pursuant to the FD&C Act.

C. Coordination of Activities

To ensure the continued coordinated regulatory, compliance, and enforcement activities for liquid chemical germicides intended for use on devices, an EPA/FDA interagency committee is established. The Directors of the EPA's Registration Division and the Compliance Division, Office of Prevention, Pesticides, and Toxic Substances, and of FDA's Center for Devices and Radiological Health, Office of Compliance and Surveillance, will serve as joint chairpersons who will designate their respective

agency members of the committee. The committee will meet at a
minimum of twice each fiscal year.

V. FUTURE RULEMAKINGS TO ELIMINATE DUPLICATIVE AGENCY REVIEW

EPA will initiate a rulemaking proceeding under section 25(b) of FIFRA to exempt liquid chemical sterilant products from regulation under FIFRA. EPA believes that the efficacy data requirements and product performance standards for liquid chemical sterilants are fulfilled by FDA's section 510(k) requirements or premarket approval requirements. When such exemption becomes effective, FDA and EPA will cease to follow procedures described in Paragraph IV, A.3. and these products will be subject solely to the regulatory and enforcement requirements and procedures of FDA, and EPA will no longer register such products. To the extent EPA receives information regarding such products, it will share such information with FDA.

FDA will initiate a rulemaking proceeding to classify liquid chemical germicides used on devices under section 513 of the FD&C Act. FDA believes that EPA's requirements under FIFRA for liquid chemical germicides that are intended for use on medical devices that are not critical or semicritical devices parallel the FD&C Act's requirements under section 510(k) of the Act. Accordingly, FDA will recommend to its classification advisory panel that liquid chemical germicides intended for use on devices that are not critical or semicritical devices be exempted from premarket notification requirements under section 510(k) of the FD&C Act. When any such exemption becomes effective, FDA and EPA will cease to follow the procedures in paragragh IV. A. 4. To the extent FDA obtains any information regarding such products, it will share the information with EPA.

VI. NAME AND ADDRESS OF PARTICIPATING PARTIES

A. Food and Drug Administration
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B. Environmental Protection Agency
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VII. LIAISON OFFICERS

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Washington, DC 20460
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VIII. PERIOD OF AGREEMENT

This agreement becomes effective upon acceptance by
both parties. It may be modified by mutual written consent or
terminated by either party upon a thirty (30) day advance
written notice to the other party. The parties agree to
evaluate the agreement every three (3) years, at which time
either party would have the option of renewing, modifying,
or canceling the agreement.

APPROVED AND ACCEPTED FOR THE APPROVED AND ACCEPTED FOR THE
ENVIRONMENTAL PROTECTION AGENCY FOOD AND DRUG ADMINISTRATION

By /Signed/ By /Signed/
Victor J. Kimm Ronald S. Chessmore

Title Acting Assistant Administrator Title Associate Commisioner for
Regulatory Affairs

Date June 4, 1993 Date June 4, 1993

Reference #3

FDA Submission - SUMMARY OF EFFICACY TESTING OF SPECTRICEPT PRODUCT VERSUS HOCI

SUMMARY OF EFFICACY TESTING OF SPECTRICEPT PRODUCT VERSUS HOCl and 3 Salts of Iron Chloride, Copper Chloride and Zinc Chloride

CONFIDENTIAL

REPORT NO. AVL-1066-R

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This report summarizes the comparison of HOCL (without the use of the 3 salts) and the Spectricept product, which contains 3 salts of copper chloride, zinc chloride and iron chloride. We utilized suspension time-kill assay as worst-case scenario by direct inoculation of each product with 6-log bacteria.

Under the test conditions for results in Table 1, the acceptance criteria were for each product to demonstrate total elimination of all microbes and achieve 6-log reduction using MRSA. This test utilizes 6-log bacteria in the presence of an added 5% serum with 15 minutes of exposure time.

Under the test conditions for results in Table 2, the acceptance criteria were for each product to demonstrate total elimination of all microbes and achieve 6-log reduction using Staphylococcus epidermidis. This test utilizes 6-log bacteria in the presence of an added 5% serum with 5 minutes of exposure time. The shorter exposure time was used in the test as an assay for determining the synergetic effects of combined salts with HOCl.

Table 1: Spectricept and HOCL tested against *MRSA* and 5% serum.

#	Formulation Name	Lot Number	FAC	Log Reduction	Pass/Fail
1	Pure (HOCL) Hypochlorous acid (8oz.)	040219	306ppm	<6 log	Fail
2	HOCl + 80ppm A, B, C	L031319-1-1C (4of20)	255ppm	≥6 log	Pass

Table 2: The efficacy of the solution with the three Salts at 75ppm, 80ppm and 100ppm with HOCl against Staphylococcus epidermidis.

#	Formulation Name	FAC	Log Reduction	Pass/Fail
1	HOCl + 100ppm of three salts	324ppm	$\geq$6 log	Pass
2	HOCl + 80 ppm of three salts	255ppm	$\geq$6 log	Pass
3	HOCl + 75ppm of three salts	327ppm	<6 log	Fail

Conclusion:

The test results summarized in Table 1 demonstrates that HOCl without the use of the three salts of copper chloride, iron chloride and zinc chloride fail to eradicate pathogens in presence of serum while the combination of HOCl with the three added salts provide full protection (6 log reduction) for the solution against pathogens in presence of serum.

Additionally, the information in table 2 demonstrates the scientific rationale for use of the three salts at 80 ppm. The test information shows the combined salts only at 80ppm or higher concentrations are effective in stabilizing HOCl in presence of organic load.

Reference #4

US Environmental Protection Agency Office of Pesticide Programs - UDM Performance Standard Revision Document

US Environmental Protection Agency
Office of Pesticide Programs

UDM Performance Standard Revision Document

November 26, 2013

November 26, 2013

Revisions to the Performance Standard for the AOAC Use-dilution Methods for *Staphylococcus aureus* (955.15) and *Pseudomonas aeruginosa* (964.02)

Background:

The AOAC Use-dilution methods (UDM) 955.15 (*Staphylococcus aureus*) and 964.02 (*Pseudomonas aeruginosa*) are laboratory assays used to measure the antimicrobial efficacy of liquid disinfectants on inanimate surfaces. Products must pass tests of both microbes for a hospital disinfectant claim. The UDM's performance standard is defined by the maximum number of positive carriers out of 60 per test per microbe to achieve a passing outcome for a product. Historically, up to one positive carrier out of 60 tested has been the performance standard for both microbes. A reassessment of the method's performance standard was conducted utilizing the best available data and statistical methodology to analyze the UDM's variability. To support revisions to the AOAC International (AOAC) standard methods, the EPA submitted three manuscripts to the Journal of AOAC International (JAOAC) for technical review and approval. The manuscripts described: 1) the outcome of a 2009 UDM collaborative study, 2) a statistical model for assessing performance standards of disinfectant test methods, and 3) the use of statistical modeling to reassess the UDM performance standard. All three manuscripts have been accepted for publication. On August 23, 2013, AOAC approved the new performance criteria for methods 955.15 and 964.02 as first action revisions to the AOAC standards. The revised AOAC methods are posted online at:
http://www.aoac.org/imis15_prod/AOAC/Default.aspx.

Reassessment Methodology:

Using the statistical model, the published variability of the UDM (based on a 2009 study) was used to reassess the performance standard. The analysis focused on an assessment of error rates, both *pass-error* and *fail-error* rates. A pass-error occurs when the disinfectant being tested has low efficacy, but is deemed a pass by the performance standard, while a fail-error occurs when the disinfectant is of acceptable efficacy, but is deemed a fail by the performance standard. The goal was to reduce error rates while maintaining a practical level of testing**.** The variability exhibited by *P. aeruginosa* was higher compared to *S. aureus*, thus the performance standards are different for each microbe. The Agency will monitor the outcome of the new performance standard criteria as data are generated, and if necessary, adjust the performance standard at a later date.

New Performance Standard Criteria:

The current version of AOAC Methods 955.15 and 964.02 posted by AOAC on September, 19, 2013 should be used for testing. Refer to the Product Performance Test Guidelines (810.2200) for efficacy testing recommendations. For a hospital disinfectant product to be deemed effective, the following criteria apply:

- Each microbe should be tested three times. Each test should be conducted against a separate batch of product for a total of three batches. All three batches should be at the lower certified limit (LCL) of the active ingredient(s). Each of the three tests

should be conducted on a different day. Testing at a single lab is acceptable. Thus, a total of three tests for *S. aureus* and three tests for *P. aeruginosa* are necessary. Sixty carriers are required per test, without contamination in the subculture media.
- The performance standard for *S. aureus* is 0-3 positive carriers out of sixty.
- The performance standard for *P. aeruginosa* is 0-6 positive carriers out of sixty.
- To be deemed an effective product, the product must pass all tests for both microbes.

Applicability:

The new performance standard criteria only apply to liquid products tested with the UDM against *S. aureus* and *P. aeruginosa*. For limited and broad spectrum disinfectant claims, the new performance standard for *S. aureus* applies. UDM requirements for testing *Salmonella enterica* are not impacted. The new performance standard criteria are applicable to data used to support new registrations, label amendments, data call-ins issued by the Agency, and in post registration testing.

References:

1. Official Methods of Analysis (2013) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, AOAC Use-dilution Method 955.15
2. Official Methods of Analysis (2013) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, AOAC Use-dilution method 964.02
3. Office of Chemical Safety and Pollution Prevention Test Guidelines: Series 810 – Product Performance Test Guidelines. See: http://www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series810.htm
4. Tomasino, S.F., Parker, A.E., Hamilton, M.A., & Hamilton, G.C. (2012) Performance of the AOAC Use-dilution method with Targeted Modifications: Collaborative Study. *J. AOAC Int.* **95** (*6*), 1618-1628
5. Parker, A.E., Hamilton, M.A., & Tomasino, S.F. (2013) A Statistical Model for Assessing Performance Standards for Quantitative and Semi-quantitative Disinfectant Test Methods. *J. AOAC Int.* (accepted for publication)
6. Tomasino, S.F., Parker, A.E., & Hamilton, M.A. (2013) Use of Statistical Modeling to Reassess the Performance Standard for the AOAC Use-dilution Methods (955.15 and 964.02). *J. AOAC Int.* (accepted for publication)
7. *Standard Operating Procedure for AOAC Use Dilution Method for Testing Disinfectants (MB-05)*, EPA Office of Pesticide Programs, Microbiology Laboratory, Environmental Science Center, Ft. Meade, MD, http://www.epa.gov/pesticides/methods/atmpa2z.htm

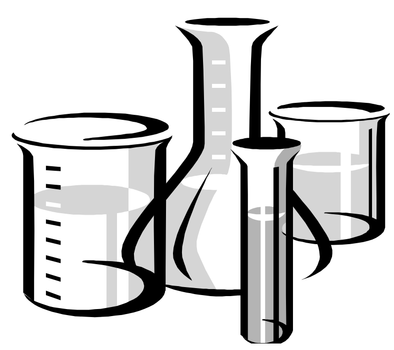
Reference #5

Product Performance Test Guidelines - OCSPP 810.2200: Disinfectants for Use on Hard Surfaces—Efficacy Data Recommendations

United States Environmental Protection Agency
Office of Chemical Safety and Pollution Prevention (7510P)
EPA 712-C-07-074
September 4, 2012

Product Performance Test Guidelines

OCSPP 810.2200: Disinfectants for Use on Hard Surfaces—Efficacy Data Recommendations



NOTICE

This guideline is one of a series of test guidelines established by the Office of Chemical Safety and Pollution Prevention (OCSPP) (formerly the Office of Prevention, Pesticides and Toxic Substances (OPPTS) prior to April 22, 2010), United States Environmental Protection Agency for use in testing pesticides and chemical substances to develop data for submission to the Agency under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601, et seq.), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, et seq.), and section 408 of the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346a), referred to hereinafter as the harmonized test guidelines.

The harmonized test guidelines serve as a compendium of accepted scientific methodologies and protocols that are intended to provide data to inform regulatory decisions under TSCA, FIFRA, and/or FFDCA. This document provides guidance for conducting appropriate tests, and is also used by EPA, the public, and the companies that are subject to data submission requirements under TSCA, FIFRA and/or the FFDCA. At places in this guidance, the Agency uses the word “should.” In this guidance, use of “should” with regard to an action means that the action is recommended rather than mandatory. As a guidance document, these guidelines are not binding on either EPA or any outside parties, and the EPA may depart from the guidelines where circumstances warrant and without prior notice. The methods contained in this guideline are strongly recommended for generating the data that are the subject of the guideline, but EPA recognizes that departures may be appropriate in specific situations. You may propose alternatives to the methods recommended in these guidelines, with your supporting rationale. The Agency will assess such proposals on a case-by-case basis.

For additional information about OCSPP harmonized test guidelines and to access the guidelines electronically, please go to http://www.epa.gov/ocspp and select “Test Methods & Guidelines” on the left side navigation menu. You may also access the guidelines in http://www.regulations.gov grouped by Series under Docket ID #s: EPA-HQ-OPPT-2009-0150 through EPA-HQ-OPPT-2009-0159, and EPA-HQ-OPPT-2009-0576.

OCSPP 810.2200: Disinfectants for use on hard surfaces - efficacy data recommendations

(a) Scope.

(1) Applicability. This guideline describes test methods that EPA believes will generally satisfy testing requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)(7U.S.C. 136, et seq.) and the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 346a). It addresses testing to demonstrate the effectiveness of antimicrobial pesticides bearing claims as disinfectants, fungicides, virucides, and tuberculocides.

(2) Background. The source materials used in developing this OCSPP test guideline are OPP guidelines 91-2: Products for use on hard surfaces and 91-30: Acceptable methods (Pesticide Assessment Guidelines, Subdivision G, Product Performance. EPA report 540/9-82-026, October 1982).

(b) Purpose. This guideline addresses efficacy testing for antimicrobial pesticides intended to be used on hard surfaces, namely disinfectants, fungicides, virucides, and tuberculocides in a variety of product types (water-soluble powders, liquids, sprays, towelettes, etc.).

(c) General considerations

(1) This guideline recommends methods for use in tests to be conducted to address the data requirements for pesticide registration. Good Laboratory Practice Standards (GLP) as defined in 40 CFR Part 160 apply to studies to support disinfection on hard, non-porous surfaces. According to 40 CFR §160.17: "EPA may refuse to consider reliable for purposes of supporting an application for a research or marketing permit any data from a study which was not conducted in accordance with this part." 40 CFR §160.12 (b) requires with any submitted research data "[a] A statement that the study was conducted in accordance with this part; [b] A statement describing in detail all differences between the practices used in the study and those required by this part; or [c] A statement that the person was not a sponsor of the study, did not conduct the study, and does not know whether the study was conducted in accordance with this part." Note: The Association of Official Analytical Chemicals (AOAC) recommended tests are designed to be conducted as written. For deviations (e.g., cultures grown with shaking instead of static, dilution of culture prior to drying on carriers) proposed to be used in the conduct of these tests, obtain written approval from the Agency and document such deviations in the study reports submitted to the Agency. The Agency may consult with the AOAC prior to accepting modifications to their standardized methods. Refer to OCSPP Test Guideline 810.2000 for general testing recommendations prior to initiating tests.

(2) Confirmatory testing. In certain situations an applicant may rely on previously submitted efficacy data to support an application or amendment for registration of a product and submit only confirmatory efficacy data on his own product to demonstrate his ability to produce

an effective formulation. These situations are as listed in paragraphs (C)(3)(i) through (C)(3)(iii) of this guideline:

(i) Duplicated Product Formulations. In this situation, the applicant manufactures a formulation which duplicates a product that is already registered with complete supporting efficacy data. The chemical composition, manufacturing procedure, label claims, and directions for use are identical in substance to those of the original registration, and specific references (Master Record ID Numbers [MRID]) to the supporting data developed for the original product are cited by the applicant.

(ii) Minor Formulation Change in a Registered Product. In this situation, the change in the formulation is relatively minor, e.g., a change of an inert ingredient. The label claims and directions for use are unchanged from those accepted for the registered formulation, and specific references (MRID) to the supporting data developed for the original formulation are cited by the applicant. If the only change in the formulation is the addition of a fragrance or dye, confirmatory data do not need to be submitted. However, when the product is an aerosol formulation, confirmatory data should be submitted for all formulation changes, including the addition of fragrances and dyes.

(iii) The confirmatory data are to be developed from testing the applicant's own finished product. When the test methodology utilized in deriving the original supporting efficacy data were modified to include additional elements not specified in the recommended method, such as organic soil, hard water, longer or shorter contact time, etc., the confirmatory data should be produced under similarly modified conditions.

(4) Efficacy claims. Table 1 provides a quick reference guide to testing for basic claims described in this guideline. Consult the text for detailed testing descriptions.

Table 1. Testing for basic efficacy claims

Level of Efficacy	Test Methods		Test Organisms	No. of Batches/Carriers	Evaluation of Success
Limited spectrum disinfectant/hard non-porous surfaces.	Water soluble powders/liquids	AOAC Use-Dilution Method or AOAC Hard Surface Carrier Test (distilled water only)	*Staphylococcus aureus* (ATCC 6538) or *Salmonella enterica* (ATCC 10708)	Three batches, one at least 60 days old. 60 carriers against either organism claimed (180 carriers).	59/60 carriers are negative for each batch tested for all methods except AOAC Hard Surface Carrier Test, which is 58/60 carriers are negative for each batch.
	Spray products	AOAC Germicidal Spray Products Test			
	Towelettes	Modified AOAC Germicidal Spray Products Test			
Broad-spectrum disinfectant/hard	Water soluble powders/liquids	AOAC Use-Dilution	*Staphylococcus aureus* (ATCC	Three batches, one at least 60 days old. 60 carriers	59/60 carriers are negative for each

Level of Efficacy	Test Methods		Test Organisms	No. of Batches/Carriers	Evaluation of Success
non-porous surfaces.		Method or AOAC Hard Surface Carrier Test (distilled water only)	6538)and *Salmonella enterica (ATCC 10708)*	against each organism (360 carriers).	batch tested for all methods except AOAC Hard Surface Carrier Test, which is 58/60 carriers are negative for each batch.
	Spray products	AOAC Germicidal Spray Products Test			
	Towelettes	Modified Germicidal Spray Test			
Hospital or healthcare disinfectant/hard non-porous surfaces.	Water soluble powders/liquids	AOAC Use-Dilution Method or AOAC Hard Surface Carrier Test (distilled water only)	*Staphylococcus aureus* (ATCC 6538)and *Pseudomonas aeruginosa* (ATCC 15442)	Three batches, one at least 60 days old. 60 carriers against each organism (360 carriers).	59/60 carriers are negative for each batch tested for all methods except AOAC Hard Surface Carrier Test, which is 58/60 carriers are negative against *Staphylococcus aureus* for each batch, and 57/60 carriers are negative against *Pseudomonas aeruginosa*.
	Spray products	AOAC Germicidal Spray Products Test			
	Towelettes	Modified Germicidal Spray Test			
Fungicidal disinfectant/hard non-porous surfaces.	Water soluble powders/liquids	AOAC Use-Dilution Test modified for fungi or AOAC Fungicidal Test	*Trichophyton mentag*rophytes (ATCC 9533)	Two batches, ten carriers per batch for the modified AOAC Use Dilution Test, the modified AOAC Germicidal Spray Products Test, and the EPA Towelette Test. Two batches for the AOAC Fungicidal Test.	All fungal spores on all carriers should be killed. For the AOAC Fungicidal Test, all fungal spores should be killed at 10 and 15 minutes to support a 10 minute label claim.
	Spray products	AOAC Germicidal Spray Products Test modified for fungi			
	Towelettes	Modified Germicidal Spray Test			
Virucidal disinfectant/hard non-porous surfaces.	Water soluble powders/liquids	AOAC Use-Dilution Test modified for viruses or ASTM E1053-	Virus claimed on the label or approved surrogate.	Two batches. One surface per batch.	Complete inactivation of the virus. Where cytotoxicity is present, demonstrate a 3 $\log_{10}$ reduction.
	Spray products	AOAC Germicidal Spray Products Test modified for viruses or ASTM E1053-			
	Towelettes	Modified Germicidal Spray Test			
Tuberculocidal disinfectant/hard non-porous	Water soluble powders/liquids	AOAC Tuberculocidal Activity of		Two batches, ten carriers per batch.	10/10 carriers are negative for growth and there

Level of Efficacy	Test Methods		Test Organisms	No. of Batches/Carriers	Evaluation of Success
surfaces.		Disinfectants, Quantitative Tuberculocidal Activity Test	*Mycobacterium bovis* BCG	Two batches, 4 replicates per batch.	is no growth in the additional test media. Survival Curve constructed from 4 separate replicates at the 95% confidence level to show probability of one survivor.
	Spray products	AOAC Germicidal Spray Products Test modified for tuberculocidal activity		Two batches, ten carriers per batch.	10/10 carriers are negative for growth and there is no growth in the additional test media.
	Towelettes	Modified Germicidal Spray Test		Two batches, ten carriers per batch.	10/10 carriers are negative for growth and there is no growth in the additional test media.
Additional bacteria/hard non-porous surfaces.	Water soluble powders/liquids	AOAC Use-Dilution Test or AOAC Hard Surface Carrier Test (distilled water only)	Bacteria claimed on the label in addition to the base broad-spectrum claim.	Two batches, ten carriers for each batch.	10/10 carriers are negative for growth of the test organism.
	Spray products	AOAC Germicidal Spray Products Test			

(d) Disinfectants

(1) Limited spectrum products. This section addresses efficacy testing for disinfectant products with limited efficacy (effective against Gram-negative or Gram-positive bacteria, but not both).

(i) Water-soluble powders and non-volatile liquid products test procedure**.** The Agency recommends the use of the AOAC International Use-Dilution Methods (Ref. 1) or the AOAC International Hard Surface Carrier Test Methods (distilled water only)(Ref. 2). Sixty carriers for each of three samples, representing three different batches, one of which should be ≥60 days old, should be tested against *Salmonella enterica* (*S. enterica*)(formerly designated as *Salmonella choleraesuis*)(American Type Culture Collection)(ATCC 10708) for effectiveness against Gram-negative bacteria, or *Staphylococcus aureus* (*S. aureus*)(ATCC 6538) for effectiveness against Gram-positive bacteria. If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(ii) Germicidal spray products (aerosol or pump) and volatile liquid products test procedure. The Agency recommends use of the AOAC International Germicidal Spray Products as Disinfectants test (Ref. 3). Sixty carriers for each of three samples, representing three different batches, one of which should be ≥60 days old, should be tested against *S. enterica* (ATCC 10708) for effectiveness against Gram-negative bacteria, or *S. aureus* (ATCC 6538) for effectiveness against Gram-positive bacteria. If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(iii) Single-use towelettes test procedure. The Agency recommends the use of a modified AOAC Germicidal Spray Products as Disinfectants test (Ref. 3) or ASTM E2362 (Ref 4). Sixty carriers for each of three samples, representing three different batches, one of which should be ≥60 days old, should be tested against *S. enterica* (ATCC 10708) for effectiveness against Gram-negative bacteria, or *S. aureus* (ATCC 6538) for effectiveness against Gram-positive bacteria. If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. Instead of spraying the inoculated surface of the carriers, the product should be tested by wiping the surface of the carriers with the saturated towelette, and then subculturing the carriers after the specified holding time. The towelette should be removed from its container and handled with sterile gloves. One towelette should be used to wipe a minimum of 10 inoculated carriers for a total of 6 towelettes for all 60 carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping a set of carriers. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(iv) Evaluation of limited disinfectant success. For the AOAC International Use-Dilution Methods, the Germicidal Spray Products as Disinfectants test, and single-use towelettes, the product should kill the test microorganisms on 59 out of each set of 60 carriers/slides in≤ ten minutes. In addition, per the 2009 AOAC revisions for the Use-Dilution Method, the mean log density for *S. aureus* is to be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6); a mean log density <6.0 invalidates the test. For the AOAC International Hard Surface Carrier Test Methods, the product should kill the test microorganisms on 58 out of each set of 60 carriers for *S. enterica* or *S. aureus* in ≤ten minutes. For the Hard Surface Carrier Test, the dried carrier counts should be $0.5 – 2.0 \times 10^6$ for *Salmonella enterica* and $1 – 5 \times 10^6$ for *Staphylococcus aureus*.

(2) Confirmatory testing for limited spectrum products

(i) Water-soluble powders and non-volatile liquid products test procedure. The Agency recommends the use of the AOAC International Use-Dilution Methods (Ref.1) or the AOAC International Hard Surface Carrier Test Methods (distilled water only) (Ref. 2). Ten carriers for each of two product samples, representing two different batches of the product, should be tested

against either *S. aureus* (ATCC 6538) or *S. enterica* (ATCC 10708)(depending on whether the product is claimed to be effective against Gram-positive or Gram-negative bacteria). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(ii) Germicidal spray products (aerosol or pump) and volatile liquid products test procedure. The Agency recommends the AOAC International Germicidal Spray Products as Disinfectants test (Ref. 3). Ten carriers for each of two product samples, representing two different batches of the product, should be tested against either *S. aureus* (ATCC 6538) or *S. enterica* (ATCC 10708)(depending on whether the product is claimed to be effective against Gram-positive or Gram-negative bacteria). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. For pressurized spray products, certification should be furnished specifying that all parts and materials used in manufacturing the container for pressurized spray disinfectants are identical to those specified by the basic manufacturer.

(iii) Single-use towelettes test procedure. The Agency recommends the use of a modified AOAC Germicidal Spray Products as Disinfectants test (Ref. 3) or ASTM E2362 (Ref 4). Ten carriers for each of two samples, representing two different batches, should be tested against *S. enterica* (ATCC 10708) for effectiveness against Gram-negative bacteria, or *S. aureus* (ATCC 6538) for effectiveness against Gram-positive bacteria. If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. Instead of spraying the inoculated surface of the carriers, the product should be tested by wiping the surface of the carriers with the saturated towelette, and then subculturing the carriers after the specified holding time. The towelette should be removed from its container and handled with sterile gloves. One towelette should be used to wipe a minimum of 10 inoculated carriers for a total of 1 towelette for all 10 carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping a set of carriers. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(iv) Evaluation of confirmatory limited disinfectant success. The product should kill all the test microorganisms on all carriers in ≤ten minutes. In addition, per the 2009 AOAC revisions for the Use-Dilution Method, the mean log density for *S. aureus* is to be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6); a mean log density <6.0 invalidates the test. For the Hard Surface Carrier Test, the dried carrier counts should be $0.5 – 2.0 \times 10^6$ for *Salmonella enterica* and $1 – 5 \times 10^6$ for *Staphylococcus aureus*.

(3) General or broad spectrum efficacy products. When a disinfectant is represented in

labeling as having efficacy against both Gram-negative and Gram-positive bacteria, the product is considered a general or broad spectrum disinfectant.

(i) Water-soluble powders and non-volatile liquid products test procedure. The Agency recommends the use of the AOAC International Use-Dilution Methods (Ref. 1) or the AOAC International Hard Surface Carrier Test Methods (distilled water only)(Ref. 2). Sixty carriers for each of three samples, representing three different batches, one of which should be ≥60 days old, should be tested against both *S. enterica* (ATCC 10708) and *S. aureus* (ATCC 6538). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(ii) Germicidal spray products (aerosol or pump) and volatile liquid products test procedure. The Agency recommends the AOAC International Germicidal Spray Products as Disinfectants test (Ref. 3). Sixty carriers for each of three samples, representing three different batches, one of which should be ≥60 days old, should be tested against both *S. enterica* (ATCC 10708) and *S. aureus* (ATCC 6538). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(iii) Single-use towelettes test procedure. The Agency recommends the use of a modified AOAC Germicidal Spray Products as Disinfectants test (Ref. 3) or ASTM E2362 (Ref 4). Sixty carriers for each of three samples, representing three different batches, one of which should be ≥60 days old, should be tested against *S. enterica* (ATCC 10708) for effectiveness against Gram-negative bacteria, and *S. aureus* (ATCC 6538) for effectiveness against Gram-positive bacteria. If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. Instead of spraying the inoculated surface of the carriers, the product should be tested by wiping the surface of the carriers with the saturated towelette, and then subculturing the carriers after the specified holding time. The towelette should be removed from its container and handled with sterile gloves. One towelette should be used to wipe a minimum of 10 inoculated carriers for a total of 6 towelettes for all 60 carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping a set of carriers. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(iv) Evaluation of general or broad spectrum disinfectant success. For the AOAC International Use-Dilution Methods, the Germicidal Spray Products as Disinfectants test, and single-use towelettes, the product should kill the test microorganisms on 59 out of each set of 60 carriers/slides in ≤ten minutes. In addition, per the 2009 AOAC revisions for the Use-Dilution Method, the mean log density for *S. aureus* is to be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6); a mean log density <6.0 invalidates the test. For the AOAC

International Hard Surface Carrier Test Methods, the product should kill the test microorganisms on 58 out of each set of 60 carriers in ≤ten minutes. For the Hard Surface Carrier Test, the dried carrier counts should be 0.5 – 2.0 x 10^6 for *Salmonella enterica* and 1 – 5 x 10^6 for *Staphylococcus aureus*.

(4) Confirmatory testing for general or broad spectrum products

(i) Water-soluble powders and non-volatile liquid products test procedure. The Agency recommends the use of the AOAC International Use-Dilution Methods (Ref. 1) or the AOAC International Hard Surface Carrier Test Methods (distilled water only)(Ref. 2). Ten carriers for each of two product samples, representing two different batches of the product, should be tested against both *S. aureus* (ATCC 6538) and S. *enterica* (ATCC 10708). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(ii) Germicidal spray products (aerosol or pump) and volatile liquid products test procedure. The Agency recommends the AOAC International Germicidal Spray Products as Disinfectants test (Ref. 3). Ten carriers for each of two product samples, representing two different batches of the product, should be tested against both *S. aureus* (ATCC 6538) and S. *enterica* (ATCC 10708). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. For pressurized spray products, certification should be furnished specifying that all parts and materials used in manufacturing the container for pressurized spray disinfectants are identical to those specified by the basic manufacturer.

(iii) Single-use towelettes test procedure. The Agency recommends the use of a modified AOAC Germicidal Spray Products as Disinfectants test (Ref. 3) or ASTM E2362 (Ref 4). Ten carriers for each of two samples, representing two different batches, should be tested against both *S. enterica* (ATCC 10708) and *S. aureus* (ATCC 6538). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. Instead of spraying the inoculated surface of the carriers, the product should be tested by wiping the surface of the carriers with the saturated towelette, and then subculturing the carriers after the specified holding time. The towelette should be removed from its container and handled with sterile gloves. One towelette should be used to wipe a minimum of 10 inoculated carriers for a total of 1 towelette for all 10 carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping a set of carriers. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(iv) Evaluation of confirmatory general or broad spectrum disinfectant success. The product should kill all the test microorganisms on all carriers in ≤ten minutes. In addition, per the

2009 AOAC revisions for the Use-Dilution Method, the mean log density for *S. aureus* is to be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6); a mean log density <6.0 invalidates the test. For the Hard Surface Carrier Test, the dried carrier counts should be $0.5 - 2.0 \times 10^6$ for *Salmonella enterica* and $1 - 5 \times 10^6$ for *Staphylococcus aureus*.

(5) Hospital or healthcare disinfectants. This section addresses efficacy testing for products recommended for use in hospitals, clinics, dental offices, nursing homes, sickrooms, or any other healthcare-related facility.

(i) Water-soluble powders and non-volatile liquid product test procedure. The Agency recommends the use of the AOAC International Use-Dilution Methods (Ref. 1) or the AOAC International Hard Surface Carrier Test Methods (distilled water only)(Ref. 2). Sixty carriers for each of three samples, representing three different batches, one of which should be ≥60 days old, should be tested against *S. aureus* (ATCC 6538), and *Pseudomonas aeruginosa* (*P. aeruginosa*)(ATCC 15442). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(ii) Germicidal spray products (aerosol or pump) and volatile liquid products test procedure. The Agency recommends the use of the AOAC International Germicidal Spray Products as Disinfectants test (Ref. 3). Sixty carriers for each of three samples, representing three different batches, one of which should be ≥60 days old, should be tested against: *S. aureus* (ATCC 6538), and *P. aeruginosa* (ATCC 15442). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(iii) Single-use towelettes test procedure. The Agency recommends the use of a modified AOAC Germicidal Spray Products as Disinfectants test (Ref. 3) or ASTM E2362 (Ref. 4). Sixty carriers for each of three samples, representing three different batches, one of which should be ≥60 days old, should be tested against *S. aureus* (ATCC 6538), and *P. aeruginosa* (ATCC 15442). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. Instead of spraying the inoculated surface of the carriers, the product should be tested by wiping the surface of the carriers with the saturated towelette, and then subculturing the carriers after the specified holding time. The towelette should be removed from its container and handled with sterile gloves. One towelette should be used to wipe a minimum of 10 inoculated carriers for a total of 6 towelettes for all 60 carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping a set of carriers. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(iv) Evaluation of hospital or healthcare disinfectant success. For the AOAC International

Use-Dilution Methods, the Germicidal Spray Products as Disinfectants test, and single-use towelettes, the product should kill the test microorganisms on 59 out of each set of 60 carriers in ≤ten minutes. In addition, per the 2009 AOAC revisions for the Use-Dilution Method, the mean log density for *S. aureus* and *P. aeruginosa* is to be at least 6.0 (corresponding to a geometric mean density of 1.0 x 10^6); a mean log density <6.0 invalidates the test. For the AOAC International Hard Surface Carrier Test Methods, the product should kill the test microorganisms on 58 out of each set of 60 carriers for *S. aureus*, and 57 out of each set of 60 carriers for *P. aeruginosa* within ten minutes or less. For the Hard Surface Carrier Test, the dried carrier counts should be 1 – 5 x 10^6 for both *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

(6) Confirmatory testing for products with hospital or healthcare disinfectant claim

(i) Water-soluble powders and non-volatile liquid products test procedure.The Agency recommends the use of the AOAC International Use-Dilution Methods (Ref. 1) or the AOAC International Hard Surface Carrier Test Methods (distilled water only)(Ref. 2). Ten carriers for each of two product samples, representing two different batches of the product, should be tested against *S. aureus* (ATCC 6538) *P. aeruginosa* (ATCC 15442). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(ii) Germicidal spray products (aerosol or pump) and volatile liquid products test procedure.The Agency recommends the AOAC International Germicidal Spray Products as Disinfectants test (Ref. 3). Ten carriers for each of two product samples, representing two different batches of the product, should be tested against *S. aureus* (ATCC 6538) and *P. aeruginosa* (ATCC 15442). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. For pressurized spray products, certification should be furnished specifying that all parts and materials used in manufacturing the container for pressurized spray disinfectants are identical to those specified by the basic manufacturer.

(iii) Single-use towelettes test procedure. The Agency recommends the use of a modified AOAC Germicidal Spray Products as Disinfectants test (Ref. 3) or ASTM E2362 (Ref. 4). Ten carriers for each of two product samples, representing two different batches of the product, should be tested against *S. aureus* (ATCC 6538) and *P. aeruginosa* (ATCC 15442). If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. Instead of spraying the inoculated surface of the carriers, the product should be tested by wiping the surface of the carriers with the saturated towelette, and then subculturing the carriers after the specified holding time. The towelette should be removed from its container and handled with sterile gloves. One towelette should be used to wipe a minimum of 10 inoculated carriers for a total of 1 towelette for all 10 carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping a set of carriers. Note: A detailed description of the wiping

procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(iv) Evaluation of confirmatory hospital or healthcare disinfectant success. The product should kill all the test microorganisms on all carriers in ≤ten minutes. In addition, per the 2009 AOAC revisions for the Use-Dilution Method, the mean log density for *S. aureus* and *P. aeruginosa* is to be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6); a mean log density <6.0 invalidates the test. For the Hard Surface Carrier Test, the dried carrier counts should be $1 – 5 \times 10^6$ for both *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

(7) Bridging for disinfectant towelettes. In some cases, disinfectant towelette formulations are identical to registered liquid formulations. In order to bridge efficacy data from the EPA registered bulk liquid disinfectant used to saturate a towelette or other related product form, the studies in paragraphs (d)(7)(i) and (d)(7)(ii) of this guideline should be conducted and submitted to EPA for review.

(i) Chemical Testing - Comparison of Expressed Liquid from the Towelette(s) to the EPA Registered Liquid Disinfectant Formulation to which it is being bridged: All active ingredients in the expressed liquid should be within the certified limits of the Confidential Statement of Formula of the liquid formula being referenced/bridged. The disinfectant towelettes package should be filled according to the manufacturing specifications. Excess liquid in the bulk towelette containers cannot be poured off for use in the chemical testing for bridging of the efficacy data. The liquid used in the chemical testing should only be that expressed from the towelettes. Three batches (one of which is 60 days old) should be tested. Analytical data for the active ingredients in the expressed liquid should be submitted for review.

(ii) Efficacy Testing **-** Efficacy testing should be conducted under the same testing conditions (e.g. soil load, contact time, temperature) as used for the bulk liquid testing. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report. For limited disinfectants, broad-spectrum disinfectants, and hospital disinfectants, to bridge bacterial disinfection claims:

(A) Test Procedure. The Agency recommends the use of the AOAC Germicidal Spray Products as Disinfectants test modified for towelettes, using the test organisms specified for limited, broad-spectrum or hospital disinfectant testing. Sixty carriers for each organism should be tested against three different batches of the product (one of which should be ≥60 days old). Instead of spraying the inoculated surface of the carrier, the product should be tested by wiping the surface of the carrier with the saturated towelette, and then subculturing the carriers after the specified holding time. One towelette should be used to treat 10 carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch.

(B) Evaluation of bactericidal towelette success. The product should kill the test

organism on 59 out of 60 carriers. This testing is intended to support bridging of all vegetative bacteria listed on the EPA registered liquid disinfectant used to saturate the towelette to the EPA registered towelette product.

(8) Disinfectants for Internal Toilet and Urinal Bowl Surfaces Above and Below the Water Line. This section addresses efficacy testing for products bearing label claims as disinfectants (limited, broad-spectrum, or hospital) for internal toilet and urinal bowl surfaces. Regarding water-soluble powders and non-volatile liquid products test procedure, the Agency recommends the use of the AOAC International Use-Dilution Methods (Ref. 1) modified to include a 5% organic soil challenge added to the bacterial inoculum. Sixty carriers for each of three samples, representing three different batches, one of which is ≥60 days old, should be tested against *Salmonella enterica* (ATCC 10708) or *Staphylococcus aureus* (ATCC 6538), for limited disinfectant products; *S. enterica* and *S. aureus*, for broad-spectrum disinfectant products; and *S. aureus* and *Pseudomonas aeruginosa* (ATCC 15442), for hospital disinfectant products. The contained bowl water (-96 fl oz, which represents traditional high volume toilets) should be used to calculate the appropriate use dilution for testing. The contained bowl water for low volume toilets should be measured and used to calculate the appropriate use dilution for testing.

(i) Evaluation of disinfectant success for internal toilet bowl and urinal bowl surfaces. For the AOAC International Use-Dilution Methods and the Germicidal Spray Products as Disinfectants test, the product should kill the test microorganisms on 59 out of each set of 60 carriers/slides within ten minutes or less. In addition, per the 2009 AOAC revisions for the Use-Dilution Method, the mean log density for *S. aureus* and *P. aeruginosa* is to be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6); a mean log density <6.0 invalidates the test.

(9) Additional microorganisms. This section addresses efficacy testing for limited, broad-spectrum or hospital disinfectants which bear label claims against bacteria other than *Salmonella enterica* (ATCC 10708), *Staphylococcus aureus* (ATCC 6538) or *Pseudomonas aeruginosa* (ATCC 15442).

(i) Water-soluble powders and non-volatile liquid products test procedure. The Agency recommends the use of the AOAC International Use-Dilution Methods (Ref. 1) or the AOAC International Hard Surface Carrier Test Methods (distilled water only)(Ref. 2). Ten carriers should be tested against each specific bacterium for each of two samples representing two different batches. If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(ii) Germicidal spray products (aerosol or pump) and volatile liquid products test procedure. The Agency recommends the use of the AOAC International Germicidal Spray Products as Disinfectants test (Ref. 3). Ten carriers should be tested against each specific bacterium for each of two samples representing two different batches. If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic

soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(iii) Single-use towelettes test procedure. The Agency recommends the use of a modified AOAC modified Germicidal Spray Products as Disinfectants test (Ref.3) or ASTM E2362 (Ref. 4). Ten carriers should be tested against each specific bacterium for each of two samples representing two different batches. If the product is intended to be represented as bactericidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. Instead of spraying the inoculated surface of the carrier, the product should be tested by wiping the surface of the carrier with the saturated towelette, and then subculturing the carriers after the specified holding time. The towelette should be removed from its container and handled with sterile gloves. One towelette should be used to wipe a minimum of 10 inoculated carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping a set of slides. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(iv) Evaluation of disinfectant success for additional microorganisms. The product should kill all the test microorganisms on all carriers in ≤ten minutes. The minimum carrier count to make the test valid should be 1×10^4.

(e) Disinfectants with fungicidal claims. This section addresses efficacy testing for broad-spectrum or hospital disinfectant products which bear label claims of efficacy against pathogenic fungi.

(1) Water soluble powders and non-volatile liquid products

(i) Test procedures. The Agency recommends the use of the AOAC International Fungicidal Activity of Disinfectants test (Ref. 5). The test should be conducted at 5, 10, and 15 minute exposure times. Two samples representing two different batches of the product should be evaluated for efficacy against *Trichophyton mentagrophytes* (*T. mentagrophytes)(*ATCC 9533). The inoculum employed should provide a concentration of $\geq 5 \times 10^6$ conidia/mL. If the product is intended to be represented as fungicidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5% blood serum, should be included with the fungal inoculum. The Agency also recommends the use of the AOAC International Use-Dilution Methods (Ref. 1). This test may be modified to conform to appropriate elements (e.g., media, growth conditions, etc.) in the AOAC International Fungicidal Activity of Disinfectants test. Ten carriers for each of two samples representing two different batches of the product should be evaluated against *T. mentagrophytes* (ATCC 9533). The inoculum employed should provide a concentration of $1 \times 10^4 – 1 \times 10^5$ conidia per carrier. If the product is intended to be represented as fungicidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the fungal inoculum.

(ii) Evaluation of fungicidal success. For the AOAC International Fungicidal Activity of Disinfectants test, all fungal spores at 10 and 15 minutes should be killed to support a 10 minute exposure time. For the AOAC International Use-Dilution Methods, all fungal spores on all 10 carriers should be killed in ≤ten minutes.

(2) Germicidal spray products (aerosol or pump) and volatile liquid products—(i) Test procedures. The Agency recommends the use of the AOAC International Germicidal Spray Products as Disinfectants test (Ref. 3).This test may be modified to conform to appropriate elements (e.g., media, growth conditions, etc.) in the AOAC International Fungicidal Activity of Disinfectants test. Ten carriers for each of two samples representing two different batches of the product should be evaluated against *T. mentagrophytes* (ATCC 9533). The inoculum employed should be modified to provide a concentration of $1 \times 10^4 – 1 \times 10^5$ conidia per carrier. If the product is intended to be represented as fungicidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the fungal inoculum.

(ii) Evaluation of fungicidal success. All fungal spores on all 10 carriers should be killed in ≤ten minutes.

(3) Single-Use Towelettes

(i) Test Procedure. The Agency recommends the use of a modified AOAC Germicidal Spray Products as Disinfectants test (Ref. 3) or ASTM 2362 (Ref. 4). Ten carriers for each of two samples representing two different batches of the product should be evaluated against *T. mentagrophytes* (ATCC 9533). The inoculum employed should be modified to provide a concentration of $1 \times 10^4 – 1 \times 10^5$ conidia per carrier. If the product is intended to be represented as fungicidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the fungal inoculum. Instead of spraying the inoculated surface of the carrier, the product should be tested by wiping the surface of the carrier with the saturated towelette, and then subculturing the carriers after the specified holding time. The towelette should be removed from its container and handled with sterile gloves. One towelette should be used to wipe a minimum of 10 inoculated carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping a set of slides. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(ii) Evaluation of fungicidal towelette success. All fungal spores on all 10 carriers should be killed in ≤ten minutes.

(4) Bridging for disinfectant towelettes. In some cases, disinfectant towelette formulations are identical to registered liquid formulations. In order to bridge efficacy data from the EPA registered bulk liquid disinfectant used to saturate a towelette or other related product

form, the studies in paragraphs (e)(4)(i) and (e)(4)(ii) of this guideline should be conducted and submitted to EPA for review:

(i) Chemical Testing - Comparison of Expressed Liquid from the Towelette(s) to the EPA Registered Liquid Disinfectant Formulation to which it is being bridged: All active ingredients in the expressed liquid should be within the certified limits of the Confidential Statement of Formula of the liquid formula being referenced/bridged. The disinfectant towelettes package should be filled according to the manufacturing specifications. Excess liquid in the bulk towelette containers cannot be poured off for use in the chemical testing for bridging of the efficacy data. The liquid used in the chemical testing should only be that expressed from the towelettes. Two batches should be tested. Analytical data for the active ingredients in the expressed liquid should be submitted for review.

(ii) Efficacy Testing - Efficacy testing should be conducted under the same testing conditions (e.g., soil load, contact time, temperature) as used for the bulk liquid testing. This testing allows bridging of data from the registered bulk liquid used to saturate the towel for each type of organism in this paragraph. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report. For fungicidal test procedure, the Agency recommends the use of the AOAC International Germicidal Spray Products as Disinfectants (Ref. 3) modified for fungicidal towelette testing. The test should be modified to conform to appropriate elements (e.g., media, growth conditions) in the AOAC International Fungicidal Activity of Disinfectants test. Ten carriers for each of two samples, representing two batches of the product should be evaluated against *T. mentagrophytes* (ATCC 9533) for the label recommended contact time. The inoculum employed should be at a count to achieve $1 \times 10^4 – 1 \times 10^5$ conidia per carrier. Instead of spraying the inoculated surface of the carrier, the product should be tested by wiping the surface of the carrier with the saturated towelette, and then subculturing the carriers after the specified holding time. One towelette should be used to wipe a minimum of 10 inoculated carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch.

(A) Evaluation of Fungicidal towelette success. The product should kill the test organism on all 10 carriers in ≤ten minutes.

(B) Bridging. This testing is intended to support bridging of all fungal test organisms from the EPA registered bulk liquid disinfectant used to saturate the towelette to the EPA registered towelette product.

(f) Disinfectants with virucidal claims. This section addresses efficacy testing for broad-spectrum or hospital disinfectant products that bear label claims of effectiveness against viruses. Virucidal products are intended for use on dry inanimate surfaces; therefore, virological data are usually developed by carrier methods. Each specific virus listed on the label should be tested, unless there is an acceptable surrogate for the virus. For label claims against Hepatitis B virus, Hepatitis C virus, and Norovirus, the Duck Hepatitis B virus, Bovine Viral Diarrhea virus,

and Feline Calicivirus, respectively, are currently considered acceptable surrogates for testing. Additional guidance and protocols for surrogate virus testing can be found at http://www.epa.gov/oppad001/regpolicy.htm. To simulate in-use conditions, the specific virus to be treated (or surrogate as noted in this paragraph) should be inoculated onto hard surfaces (e.g., Petri dishes, glass carriers, or other appropriate test surface), allowed to dry, and then treated with the product according to the directions for use on the product label.

(1) Water soluble powders and non-volatile liquid products test procedures. The Agency recommends the use of either the AOAC International Use-Dilution Methods (Ref 1) modified for virucidal testing or the ASTM E1053 Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces (Ref. 6). One surface for each of two samples, representing two different batches of disinfectant, should be tested against a recoverable virus end point titer of $\geq 10^4$ viable viral particles from the test surface for a specified exposure period ($\leq$10 minutes) at room temperature. If the product is intended to be represented as virucidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the viral inoculum. When viral suspensions are grown in the presence of at least 5% serum, addition of serum to the inoculum is not expected as part of a study to support a one-step label claim.

(2) Germicidal spray products (aerosol or pump) and volatile liquid products test procedure. The Agency recommends the use of a AOAC International Germicidal Spray Products as Disinfectants test (Ref. 3) modified for virucidal testing or the ASTM E1053 Virucidal Test Method (Ref. 6). One surface for each of two samples, representing two different batches of disinfectant, should be tested against a recoverable virus endpoint titer of at least 10^4 viable viral particles from the test surface for the exposure period specified on the label. If the product is intended to be represented as virucidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the viral inoculum. When viral suspensions are grown in the presence of at least 5% serum, addition of serum to the inoculum is not expected as part of a study to support a one-step label claim.

(3) Single-use towelettes test procedure. The Agency recommends the use of the modified AOAC Germicidal Spray Products as Disinfectants test (Ref. 3) or ASTM E1053 (Ref. 6). One surface for each of two samples, representing two different batches of disinfectant, should be tested against a recoverable virus end point titer of $\geq 10^4$ viable viral particles from the test surface for a specified exposure period ($\leq$10 minutes) at room temperature. If the product is intended to be represented as virucidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the viral inoculum. When viral suspensions are grown in the presence of at least 5% serum, addition of serum to the inoculum is not expected as part of a study to support a one-step label claim. Instead of spraying the inoculated surface of the carrier, the product should be tested by wiping the surface of the carrier with the saturated towelette, and then subculturing the carriers after the specified holding time. The towelette should be removed from its container and handled with sterile gloves. One towelette should be used to wipe a minimum of 10 inoculated carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per

carrier set per batch. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping a set of slides. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(4) Evaluation of virucidal success. Following treatment of the test virus with the disinfectant product, the presence of remaining viable virus should then be assayed using an appropriate virological technique (e.g., cytopathogenic effect, fluorescent antibody, plaque count, or animal response). The protocol for the viral assay should provide the information identified in paragraphs (f)(4)(i)through (f)(4)(ix) of this guideline.

(i) The virus recovery (titer) should include a minimum of four determinations per each dilution in the assay system (e.g., tissue culture, embryonated egg, animal infection, etc.).

(ii) Cytotoxicity controls. The effect of the disinfectant on the viral assay system should include a minimum of four determinations per each dilution. For EPA approved protocols for surrogate virus testing, two determinations per each dilution should be included.

(iii) The activity of the disinfectant against the test virus should include a minimum of four determinations per dilution in the assay system.

(iv) Neutralization controls. Neutralization controls should be performed (Ref. 7) and should include a minimum of four determinations per each dilution. For EPA approved protocols for surrogate virus testing, two determinations per each dilution should be included.

(v) Any special methods which are used to increase the virus titer and to detoxify the residual disinfectant should be described.

(vi) The ID50 values calculated for each assay should be provided.

(vii) The test results should be reported as the reduction of the virus titer by the activity of the disinfectant (ID_{50} of the virus control less the ID_{50} of the test system) expressed as the logarithm to the base 10 and calculated by a statistical method (e.g., Reed and Munch, Most Probable Number, Spearman-Karber).

(viii) The product should demonstrate complete inactivation of the virus at all dilutions. If cytotoxicity is present, the virus control titer should be increased to demonstrate a $\geq 3 \log_{10}$ reduction in viral titer beyond the cytotoxic level. Table 1 provides an example of a typical laboratory report of a single test with one virus, assayed in a tissue culture system.

(ix) A laboratory report of a single test with one virus (recovered from a treated surface) involving a tissue culture assay system should include the details of the methods employed and the information included in Tables 2-1, 2-2 and 2-3:

Table 2-1: Test Results

Dilution of Virus	Virus - Disinfectant*	Virus - Control*	Cytotoxic - Control
10^{-1}	T T T T	+ + + +	T T T T
10^{-2}	T T T T	+ + + +	T T T T
10^{-3}	T 0 0 0	+ + + +	T 0 0 0
10^{-4}	0 0 0 0	+ + + +	0 0 0 0
10^{-5}	0 0 0 0	+ + + +	0 0 0 0
10^{-6}	0 0 0 0	+ + + 0	0 0 0 0
10^{-7}	0 0 0 0	+ 0 0 0	0 0 0 0
10^{-8}	0 0 0 0	0 0 0 0	0 0 0 0

Note: T = toxic; + = virus recovered; 0 = no virus recovered

Table 2-2: Calculation of the Tissue Culture Infective Dose 50 ($TCID_{50}$)

Values				Accumulated Values			
Virus Dilution Inoculated	No. Infected / No. Inoculated	No. Infected	No. not Infected	No. Infected	No. not Infected	No. Infected / No. Inoculated	% Infected
10^{-1}	4/4	4	0	24	0	24/24	100
10^{-2}	4/4	4	0	20	0	20/20	100
10^{-3}	4/4	4	0	16	0	16/16	100
10^{-4}	4/4	4	0	12	0	12/12	100
10^{-5}	4/4	4	0	8	0	8/8	100
10^{-6}	3/4	3	1	4	1	4/5	80
10^{-7}	1/4	1	3	1	4	1/5	20
10^{-8}	0/4	0	4	0	8	0/8	0

$TCID_{50} = 10^{6.5}$

Table 2-3: Calculations of the Tissue Culture Lethal Dose 50 ($TCLD_{50}$)

Values	Accumulated Values

Virus Dilution Inoculated	No. Toxic / No. Inoculated	No. Toxic	No. not Toxic	No. Toxic	No. not Toxic	No. Toxic / No. Inoculated	% Toxic
10^{-1}	4/4	4	0	9	0	9/9	100
10^{-2}	4/4	4	0	5	0	5/5	100
10^{-3}	1/4	1	3	1	3	1/4	25
10^{-4}	0/4	0	4	0	7	0/7	0
10^{-5}	0/4	0	4	0	11	0/11	0
10^{-6}	0/4	0	4	0	15	0/15	0
10^{-7}	0/4	0	4	0	19	0/19	0
10^{-8}	0/4	0	4	0	23	0/23	0

$TCLD_{50} = 10^{2.7}$ Therefore: Virus inactivation = $TCID_{50}$ - $TCLD_{50}$ = $10^{3.8}$ log 10

(5) Bridging for disinfectant towelettes. In some cases, disinfectant towelette formulations are identical to registered liquid formulations. In order to bridge efficacy data from the EPA registered bulk liquid disinfectant used to saturate a towelette or other related product form, the studies in paragraphs (f)(5)(i) and (f)(5)(ii) of this guideline should be conducted and submitted to EPA for review.

(i) Chemical Testing—Comparison of Expressed Liquid from the Towelette(s) to the EPA Registered Liquid Disinfectant Formulation to which it is being bridged: All active ingredients in the expressed liquid should be within the certified limits of the Confidential Statement of Formula of the liquid formula being referenced/bridged. The disinfectant towelettes package should be filled according to the manufacturing specifications. Excess liquid in the bulk towelette containers cannot be poured off for use in the chemical testing for bridging of the efficacy data. The liquid used in the chemical testing should only be that expressed from the towelettes. Two batches should be tested. Analytical data for the active ingredients in the expressed liquid should be submitted for review.

(ii) Efficacy Testing: Efficacy testing should be conducted under the same testing conditions (e.g. soil load, contact time, temperature) as used for the bulk liquid testing. This testing allows bridging of data from the registered bulk liquid used to saturate the towel for each type of virus in paragraphs (f)(5)(ii)(A*)(1)* through (f)(5)(ii)(A)*(3)* of this guideline. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(A) Virucidal Test Procedure. The Agency recommends the use of either the AOAC International Germicidal Spray Products as Disinfectants (Ref. 3) modified for virucidal towelette testing or the ASTM E1053 (Ref. 6) modified for virucidal towelette testing.

(1) To support bridging of all viral claims, the most difficult to inactivate small-sized non-enveloped virus, from the viral strains registered for the bulk liquid, should be selected for testing. Examples of small-sized non-enveloped viral families include members of the Picornaviridae family (e.g., poliovirus, enterovirus, hepatitis A virus, rhinovirus), and Parvoviridae family (e.g., parvovirus).

(2) To support bridging of viral claims for large-sized non-enveloped and enveloped viral strains, the most difficult to inactivate large-sized non-enveloped virus, from the viral strains registered for the bulk liquid, should be selected for testing. Examples of large-sized non-enveloped viral families include members of the Adenoviridae family (e.g., adenovirus), Reoviridae family (e.g., rotavirus), and Papillomaviridae family (e.g., papillomavirus).

(3) To support bridging of viral claims for enveloped viral strains, the most difficult to inactivate enveloped virus, from the viral strains registered for the bulk liquid, should be selected for testing. Examples of enveloped viral families include members of the Coronaviridae family (e.g., coronavirus), Flaviviridae family (e.g., hepatitis C virus), Herpesviridae family (e.g., herpes virus), Poxviridae family (e.g., vaccinia), Hepadnaviridae family (e.g., hepatitis B virus), Orthomyxoviridae family (e.g., Influenza), Paramyxoviridae family (e.g., parainfluenza) and Retroviridae family (e.g., human immunodeficiency virus).

(B) Ten carriers for each of two samples, representing two batches of disinfectant, should be tested against a recoverable dried virus end point titer of $\geq 10^4$ viral particles from the test surface for a specified exposure period at room temperature. Instead of spraying the inoculated surface of the carrier, the product should be tested by wiping the surface of the carrier with the saturated towelette, and then subculturing the carriers after the specified holding time. One towelette should be used to wipe a minimum of 10 inoculated carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch.

The protocol for the viral assay should provide the information identified in paragraphs (f)(5)(ii)(B)*(1)* through (f)(5)(ii)(B)*(7)* of this guideline:

(1) The virus recovery (titer) should include a minimum of four determinations for each dilution in the assay system (e.g., cell culture, embryonated egg, animal infection).

(2) Cytotoxicity controls. The effect of the test substance on the viral assay system should include a minimum of four determinations for each dilution. For EPA approved protocols for surrogate virus testing, two determinations per each dilution should be included.

(3) The activity of the test substance against the test virus should include a minimum of four determinations for each dilution in the assay system.

(4) Neutralization controls. Neutralization controls should be performed (Ref. 7) and should include a minimum of four determinations per each dilution. For EPA approved protocols

for surrogate virus testing, two determinations per each dilution should be included.

(5) Any special methods which are used to increase the virus titer and to detoxify the residual test substance should be described.

(6) The LD_{50} values calculated for each assay should be provided.

(7) The test results should be reported as the reduction of the virus titer by the activity of the test substance (LD_{50} of the virus control less the LD_{50} of the test system) expressed as the logarithm to the base 10 and calculated by a statistical method (e.g., Reed and Munch, Most Probable Number, Spearman-Karber).

(C) Evaluation of virucidal success. The product should demonstrate complete inactivation of the virus at all dilutions. If cytotoxicity is present, a ≥3-log reduction in viral titer should be demonstrated beyond the cytotoxic level recovered from the carrier surface.

(g) Disinfectants with tuberculocidal claims. This section addresses efficacy testing for broad-spectrum or hospital disinfectant products which bear label claims of effectiveness as tuberculocides. In the Agency's "Data Call-In Notice for Tuberculocidal Effectiveness for All Antimicrobial Pesticides with Tuberculocidal Claims," dated June 13, 1986 (Ref. 8), applicants were given the option of choosing from one of three test methods (AOAC Tuberculocidal Activity of Disinfectants test, a modified AOAC Tuberculocidal Activity of Disinfectants test, or the Quantitative Tuberculocidal Activity Test) for conducting tuberculocidal efficacy tests. In general, the Agency does not believe that the Quantitative Tuberculocidal Activity Test (a suspension test) is appropriate for disinfectant formulations used on hard surfaces. An exception to this is for glutaraldehyde-based products, which have never been validated in the AOAC Tuberculocidal Activity of Disinfectants test (a carrier based test). Therefore, the Quantitative Tuberculocidal Activity Test should only be used for glutaraldehyde-based products. The Agency strongly recommends all other formulations to use the carrier-based AOAC Tuberculocidal Activity of Disinfectants test.

(1) Water-soluble powders and non-volatile liquid products test procedures. The Agency recommends the test procedures in paragraphs (g)(1)(i) through (g)(1)(iv) of this guideline.

(i) AOAC International Tuberculocidal Activity of Disinfectants test. The AOAC International Tuberculocidal Activity of Disinfectants test (Ref. 9) employing a 10 minute contact time and 20˚C temperature. Ten carriers for each of two samples representing two different batches of the product should be tested against *Mycobacterium bovis* (BCG)(*M.bovis*). If the product is intended to be represented as tuberculocidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. The log density of *M. bovis* should be ≥4.0 (corresponding to a geometric mean density of $\geq 1.0 \times 10^4$ CFU/carrier) a mean log density of <4.0 invalidates the test.

(ii) AOAC International Tuberculocidal Activity of Disinfectants test with modifications. The AOAC International Tuberculocidal Activity of Disinfectants test with modifications to the 10 minute contact time and/or 20˚C temperature (Ref. 9). Ten carriers for each of two samples representing two different batches of the product should be tested against *M. bovis* (BCG). If the product is intended to be represented as tuberculocidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. The log density of *M. bovis* should be ≥4.0 (corresponding to a geometric mean density of $\geq 1.0 \times 10^4$ CFU/carrier) a mean log density of <4.0 invalidates the test.

(iii) Evaluation of tuberculocide success. For the AOAC International Tuberculocidal Activity of Disinfectants test, all organisms on all carriers should be killed, and there should be no growth in any of the inoculated subculture media.

(iv) Validation testing for Quaternary Ammonium Compounds. Products formulated solely with quaternary ammonium compounds as the active ingredient(s) should be supported with validation testing to confirm their tuberculocidal label claim. One additional product sample should be tested in a different laboratory from the original one, or in the same laboratory using different study director, technical staff and quality assurance unit, using the same test procedure and conditions as used in the first laboratory test.

(2) Glutaraldehyde formulations

(i) Test Procedure. For glutaraldehyde formulations, the Agency recommends the Quantitative Tuberculocidal Activity Test. This test has been published in the Agency's "Data Call-In Notice for Tuberculocidal Effectiveness for All Antimicrobial Pesticides with Tuberculocidal Claims," dated June 13, 1986 (Ref. 8). Two samples, representing two different batches of the product should each be utilized in at least four separate studies (a total of at least eight studies), against *M.* bovis, so that upper 95 percent confidence limits can be determined for each point on the survival curve. If the product is intended to be represented as tuberculocidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum.

(ii) Evaluation of tuberculocide success. For the Quantitative Tuberculocidal Activity Method, survival curves should be constructed from the average of four separate replicates so that the upper 95% Confidence Limit can be determined for each point on the curve. The minimum time claimed for efficacy is determined by finding the point where the average survival curve intersects the probability of one survivor. If the data show a four-log reduction, but the survivor curve does not intersect the one-survivor line, the minimal time is found by extrapolating the upper 95% confidence limit curve such that the value where it intersects the one survivor line is not 50% greater than when the survivor curve intersects the one survivor line.

(3) Germicidal spray products and volatile liquid products—(i) Test procedure. The

Agency recommends the AOAC International Germicidal Spray Products as Disinfectants test (Ref. 3), using the media, test culture, and other elements described in the AOAC International Tuberculocidal Activity of Disinfectants test. Ten carriers for each of two samples representing two different batches of the product should be tested against *M. bovis* (BCG). If the product is intended to be represented as tuberculocidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. The log density of *M. bovis* should be ≥4.0 (corresponding to a geometric mean density of $\geq 1.0 \times 10^4$ CFU/carrier) a mean log density of <4.0 invalidates the test.

(ii) Evaluation of tuberculocide success. When using the AOAC International Germicidal Spray Products as Disinfectants test, all organisms on all carriers/slides should be killed, and there should be no growth in any of the inoculated subculture media.

(4) Single-Use Towelettes

(i) Test Procedure. The Agency recommends the use of a modified AOAC Germicidal Spray Products as Disinfectants test (Ref.3) or ASTM 2362 (Ref. 4). Ten carriers for each of two samples representing two different batches of the product should be evaluated against *M. bovis* (BCG). If the product is intended to be represented as tuberculocidal in the presence of organic soil (one-step), an appropriate organic soil, such as 5 percent blood serum, should be included with the bacterial inoculum. Instead of spraying the inoculated surface of the carrier, the product should be tested by wiping the surface of the carrier with the saturated towelette, and then subculturing the carriers after the specified holding time. The towelette should be removed from its container and handled with sterile gloves. One towelette should be used to wipe a minimum of 10 inoculated carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch. The area of the towelette used for wiping should be rotated so as to expose a maximum amount of its surface in the course of wiping a set of slides. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report. The log density of *M. bovis* should be ≥4.0 (corresponding to a geometric mean density of $\geq 1.0 \times 10^4$ CFU/carrier) a mean log density of <4.0 invalidates the test.

(ii) Evaluation of tuberculocidal towelette success. All organisms on all carriers should be killed, and there should be no growth in any of the inoculated subculture media.

(5) Bridging for disinfectant towelettes. In some cases, disinfectant towelette formulations are identical to registered liquid formulations. In order to bridge efficacy data from the EPA registered bulk liquid disinfectant used to saturate a towelette or other related product form, the studies in paragraphs (g)(5)(i) and (g)(5)(ii) of this guideline should be conducted and submitted to EPA for review.

(i) Chemical Testing - Comparison of Expressed Liquid from the Towelette(s) to the EPA Registered Liquid Disinfectant Formulation to which it is being bridged: All active ingredients in

the expressed liquid should be within the certified limits of the Confidential Statement of Formula of the liquid formula being referenced/bridged. The disinfectant towelettes package should be filled according to the manufacturing specifications. Excess liquid in the bulk towelette containers cannot be poured off for use in the chemical testing for bridging of the efficacy data. The liquid used in the chemical testing should only be that expressed from the towelettes. Two batches should be tested. Analytical data for the active ingredients in the expressed liquid should be submitted for review.

(ii) Efficacy Testing: Efficacy testing should be conducted under the same testing conditions (e.g. soil load, contact time, temperature) as used for the bulk liquid testing. This testing allows bridging of data from the registered bulk liquid used to saturate the towel for each type of organism in paragraph (g)(5)(ii)(A) of this guideline. Note: A detailed description of the wiping procedure, including the towelette folding and rotation process should be included in the test protocol and documented in the raw data and final report.

(A) Test Procedure. The Agency recommends the use of the modified AOAC Germicidal Spray Products as Disinfectants test (Ref. 3) or ASTM 2362 (Ref. 4). The test should be modified to conform to appropriate elements (e.g., media, growth conditions, etc) in the AOAC International Tuberculocidal Activity of Disinfectants test. Ten carriers for each of two samples, representing two batches of the product, should be tested against *M. bovis* BCG. Instead of spraying the inoculated surface of the carrier, the product should be tested by wiping the surface of the carrier with the saturated towelette, and then subculturing the carriers after the specified holding time. One towelette should be used to wipe a minimum of 10 inoculated carriers. Alternatively, one carrier with a surface area equivalent to ten 1 x 1 inch carriers can be wiped using one towelette per carrier set per batch. The log density of *M. bovis* should be ≥ 4.0 (corresponding to a geometric mean density of $\geq 1.0 \times 10^4$ CFU/carrier) a mean log density of <4.0 invalidates the test.

(B) Evaluation of tuberculocidal towelette success. All organisms on all carriers should be killed, and there should be no growth in any of the inoculated subculture media.

(C) This testing is intended to support bridging of all mycobacteria listed on the EPA registered liquid disinfectant used to saturate the towelette to the EPA registered towelette product.

(h) Data collection and reporting—(1) General. To assist in the proper review and evaluation of product performance, complete descriptions of the test employed and the results obtained should be submitted to the Agency. All test reports should include, at the least, the following information:

(i) Study title;

(ii) Product Identity;

(iii) Guideline number/Data Requirement;

(iv) Identification of the testing laboratory or organization;

(v) Location where the test was performed;

(vi) Name(s) of the person(s) responsible for the test;

(vii) Statement of Confidentiality Claims;

(viii) Statement of 40 CFR Part 160 Good Laboratory Practice compliance and Quality Assurance Statement;

(ix) Purpose of the study;

(x) Date and time of the start and end of the test;

(xi) Test employed and any modifications (e.g., organic soil, hard water, etc.), when using standard tests (e.g., AOAC, ASTM, etc.) all deviations to the test methods should be reported;

(xii) Test microorganisms employed, including identification of the specific strain (ATCC or other);

(xiii) Description of the test substance, including the percent of active ingredient;

(xiv) Concentration or dilution of the product tested and how prepared;

(xv) Number of samples, batches and replicates tested;

(xvi) Manufacture date of each product batch;

(xvii) Identification of all material or procedural options employed, where such choice is provided for or recommended in the test method selected (e.g., growth media, drying time for inoculated carriers, neutralization confirmation and/or subculture media, secondary subculturing);

(xviii) Test exposure conditions (e.g., contact time, temperature, and relative humidity);

(xix) Complete reports of results obtained for each replication;

(xx) Any control data essential to establish the validity of the test.

(xxi) Carrier counts;

(xxii) Statistical treatment of the data;

(xxiii) Conclusions;

(xxiv) References;

(xxv) Appendices, including study protocol and all raw data reports (per 40 CFR Part 160.185) associated with the conduct of the study.

The applicant is encouraged to use the EPA's standard efficacy report format, which may be found at http://www.epa.gov/oppad001/efficacystudystandards.htm.

(2) Data for modifications of recommended methods. When recommended methods are modified to support specific claims and/or use patterns for a product, the protocol, identifying and describing each modification, should be provided with the study report. The applicant should submit the proposed modification to the Agency for review and evaluation prior to initiation of the test.

(3) Data for other methods. When recommended methods, or modifications thereto, are not employed to develop efficacy data (such as actual in-use or many kinds of simulated-use testing), complete testing protocols should be submitted with the test reports. All materials and procedures employed in testing should be described in a manner consistent with original research reports published in technical or scientific journals. Where references to published reports or papers are made, copies or reprints of such references should be provided with the test reports. The applicant should submit the proposed testing protocols for in-use or simulated-use studies (with a proposed label to show the claims to be supported by the protocol) to the Agency for review and evaluation prior to initiation of the test.

(i) References. The references in this paragraph may be consulted for additional background information:

(1) *Official Methods of Analysis of the AOAC International*, Chapter 6, Disinfectants, Use-Dilution Methods (955.14, 955.15, & 964.02), Current edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417.

(2) *Official Methods of Analysis of the AOAC International,* Chapter 6, Disinfectants, Hard Surface Carrier Test Methods, Current edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417.

(3) *Official Methods of Analysis of the AOAC International,* Chapter 6, Disinfectants, Official Method 961.02 Germicidal Spray Products as Disinfectants, Current edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417.

(4) *Annual Book of ASTM Standards*, Standard Practice for Evaluation of Pre-saturated or Impregnated Towelettes for Hard Surface Disinfection, Designation E2362. American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, PA 19428, current edition.

(5) *Official Methods of Analysis of the AOAC International*, Chapter 6, Disinfectants, Official Method 955.17 Fungicidal Activity of Disinfectants. Current edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417.

(6) *Annual Book of ASTM Standards*, Test Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces, Designation E1053. American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, PA 19428, current edition.

(7) *Annual Book of ASTM Standards*, Standard Test Method for Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations, Designation E1483. American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, PA 19428, current edition.

(8) Environmental Protection Agency, Data Call-in Notice for Tuberculocidal Effectiveness Data for All Antimicrobial Pesticides with Tuberculocidal Claims (Registration Division, Office of Pesticide Programs, June 13, 1986).

(9) *Official Methods of Analysis of the AOAC International*, Chapter 6, Disinfectants, Official Method 965.12 Tuberculocidal Activity of Disinfectants. Current edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417.

Reference #6

US EPA – Office of Pesticide Programs. Standard Operating Procedure for the AOAC Sporicidal activity of disinfectant tests

US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for the AOAC Sporicidal Activity of Disinfectants Test (Bacillus subtilis × porcelain carrier)

SOP Number: MB-15-04

Date Revised: 01-03-18

SOP Number	MB-15-04
Title	Standard Operating Procedure for the AOAC Sporicidal Activity of Disinfectants Test (*Bacillus subtilis* × porcelain carrier)
Scope	This SOP describes the Sporicidal Activity of Disinfectants Test – Method II methodology used to determine the sporicidal efficacy of liquid sporicidal agents against *Bacillus subtilis* on hard surfaces (porcelain carriers).
Application	The method is based on AOAC method 966.04 (see 15.1). *B. subtilis* (ATCC #19659) is a test microbe used to support sporicidal claims. Testing of suture loops and *Clostridium sporogenes* is not addressed in this SOP.

	Approval Date
SOP Developer:	______________________________ Print Name: ______________________
SOP Reviewer	______________________________ Print Name: ______________________
Quality Assurance Unit	______________________________ Print Name: ______________________
Branch Chief	______________________________ Print Name: ______________________

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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<table>
<tr><td>1. Definitions</td><td>Additional abbreviations/definitions are provided in the text.
1. AOAC = AOAC INTERNATIONAL
2. CFU = Colony Forming Unit
3. References to water mean reagent-grade water, except where otherwise specified.</td></tr>
<tr><td>2. Health and Safety</td><td>Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Safety Data Sheet for specific hazards associated with products.</td></tr>
<tr><td>3. Personnel Qualifications and Training</td><td>Refer to SOP ADM-04, OPP Microbiology Laboratory Training.</td></tr>
<tr><td>4. Instrument Calibration</td><td>Refer to SOPs EQ-01 (pH meters), EQ-02 (thermometers), EQ-03 (weigh balances), EQ-05 (timers), EQ-10 (orbital shakers) and QC-19 (pipettes) for details on method and frequency of calibration.</td></tr>
<tr><td>5. Sample Handling and Storage</td><td>Refer to SOP MB-22, Preparation and Sampling Procedures for Antimicrobial Test Substances, and SOP COC-01, Chain of Custody Procedures.</td></tr>
<tr><td>6. Quality Control</td><td>For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).</td></tr>
<tr><td>7. Interferences</td><td>Avoid touching the interior sides of the medication tube while the carrier is being lowered into the disinfectant and the hook is being removed as it may lead to false positive results.</td></tr>
<tr><td>8. Non-conforming Data</td><td>1. Sterility and/or viability controls do not yield expected results.
2. The mean spore counts per carrier falls outside the specified range of 1×10^5 to approximately 1.0×10^6 spores/carrier.
3. No contamination is acceptable in the test system.
4. Acceptable spore purity and HCl resistance test results must be achieved.
5. Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports.</td></tr>
<tr><td>9. Data Management</td><td>Data will be archived consistent with SOP ADM-03, Records and Archives.</td></tr>
<tr><td>10. Cautions</td><td>1. To ensure the stability of a diluted sporicidal agent, use the diluted product within three hours of preparation unless specified otherwise.</td></tr>
</table>

	2. Use appropriate aseptic techniques for all test procedures involving the manipulation of the test organisms and associated test components. 3. Verify the volume of dilution blanks, neutralizer tubes, and subculture tubes in advance and adjust accordingly.
11. Special Apparatus and Materials	1. Culture Media. <u>Note</u>: Commercial dehydrated media made to conform to the specified recipes may be substituted. Media can be stored for up to two months. a. *Nutrient broth*. For use in preparing nutrient agar. Add 5 g beef extract (paste or powder), 5 g NaCl, and 10 g peptone (anatone) to approximately 1 L water. Boil mixture for 20 min with constant stirring. Readjust volume to 1 L with water and allow cooling to around 50ºC. Adjust pH to 6.8±0.2 with 1N HCl or 1N NaOH, if necessary. Filter through paper (e.g., Whatman filter paper No. 4). Dispense 10 mL portions into 20×150 mm culture tubes or 20 mL portions into 25×150 mm culture tubes. Dehydrated nutrient broth may be substituted – prepare according to the manufacturer's instructions. b. *Nutrient agar*. For stock cultures slants. Add 1.5% (w/v) Bacto-agar to unsterilized nutrient broth. Boil mixture until agar is dissolved. Adjust pH to 7.2±0.2 if necessary. Dispense 5 mL portions into 16×100 mm screw cap tubes. Larger tubes may be used as well. Autoclave for 20 min at 121ºC. Remove from autoclave and slant tubes to form agar slants. c. *Nutrient agar with 5µg/mL $MnSO_4 \cdot H_2O$ (amended nutrient agar)*. For spore production. Suspend 11.5 g nutrient agar in 495 mL water, add 5 mL 500 ppm $MnSO_4$:H_2O. Dissolve by boiling. Adjust pH to 6.8±0.2 if necessary. Autoclave for 15 min at 121ºC. Pour agar into plates. d. *Trypticase soy agar (TSA)*. Prepare according to manufacturer's instructions. e. *Fluid thioglycollate medium (FTM)*. For subculturing spores exposed to disinfectant. Prepare according to manufacturer's instructions. Protect from light. <u>Note</u>: If after autoclaving the aerated portion of media consumes more than one third of tube, media must be re-boiled by placing tubes in beaker of boiling water. Media can only be re-boiled once. f. *Fluid thioglycollate medium with 1M NaOH (modified FTM)*. For

subculturing spores exposed to 2.5 M HCl. Suspend 29.5 g of fluid thioglycollate medium in 1 L water. Heat boiling to dissolve completely. Cool and adjust pH to 7.1±0.2 if necessary. Add 20 mL 1 M NaOH, mix well. Check final pH and record (pH between 8 and 9 is typical). Dispense 10 mL into 20×150 mm culture tubes and autoclave for 15 min at 121ºC. Store at room temperature. Protect from light.

2. *500 ppm Manganese sulfate monohydrate*. Add 0.25 g manganese sulfate monohydrate to 500 mL water. Filter sterilize.
3. *2.5 M Dilute hydrochloric acid*. Use to determine resistance of dried spores. Standardize and adjust to 2.5 M as in AOAC method 936.15 or purchase certified 2.5M HCl.
4. *Sterile water*. Use reagent-grade water. Reagent-grade water should be free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. Reverse osmosis, distillation, and deionization in various combinations all can produce reagent-grade water when used in the proper arrangement. See Standard Methods for the Examination of Water and Wastewater for details on reagent-grade water.
5. *Triton X-100.* For washing used porcelain penicylinders.
6. *Ethanol (40%).* For preparing spore suspension used in the neutralization assay.
7. *3M™ Petrifilm™ Aerobic Count Plate*. For spore enumeration. 3M Food Safety (St. Paul, MD, USA; Cat. No. 6400).
8. *Test organism. Bacillus subtilis* (ATCC No. 19659) obtained directly from a reputable supplier (e.g., ATCC).
9. *Carriers.* Penicylinders, porcelain, 8±1 mm OD, 6±1 mm ID, 10±1 mm length. (Available from CeramTec Ceramic, Laurens, SC, www.ceramtec.com, SAP# 1010368)
10. *Glassware*. For disinfectant, 25×100 mm culture tubes (Bellco Glass Inc., Vineland, NJ; reusable or disposable 20×150 mm (for cultures/subcultures); 16×100 mm screw cap tubes for stock cultures. Cap with closures before sterilizing. Sterilize all glassware 2 h in hot air oven at 180°C or steam sterilize for a minimum of 20 min at 121°C with drying cycle.
11. *Sterile centrifuge tubes*. Polypropylene, 15 mL conical tubes with conical bottoms (Corning), or equivalent.

	12. *Water bath/chiller unit.* Constant temperature for test chemical, capable of maintaining 20±1°C temperature or specified temperature for conducting the test. 13. *Petri dishes*. Plastic (sterile). 14. *Filter paper*. Whatman filter paper #2; placed in Petri dishes for storing carriers. 15. *Test tube racks*. Any convenient style. 16. *Inoculating loop*. Any convenient inoculation/transfer loop for culture transfer. 17. *Wire hook*. For carrier transfer. Make 3 mm right angle bend at end of 50-75 mm nichrome wire No. 18 B&S gage. Have other end in suitable holder. 18. *Centrifuge.* For preparing spore suspension. 19. *Sonicator* (ultrasonic cleaner). For conducting control carrier counts. 20. *Orbital shaker.* For preparing spore suspension. Speed range from 25 to 500 rpm. 21. *Vacuum desiccator*. For carrier storage. With gauge for measuring 27" (69 cm) of Hg and fresh desiccant. 22. *Certified Timer*. For managing timed activities, any certified timer that can display time in seconds.
12. Procedure and Analysis	
12.1 Culture Initiation	a. Every 12 months (or sooner if necessary) initiate a new stock culture from a lyophilized culture of *B. subtilis* (ATCC 19659). b. Open ampule of freeze dried organism as indicated by ATCC. c. Using a tube containing 5-6 mL of nutrient broth (NB), aseptically withdraw 0.5 to 1.0 mL and rehydrate the pellet for *B. subtilis*. d. Aseptically transfer the entire rehydrated pellet back into the original tube of nutrient broth designated as "TUBE A" (see Attachment 1). Mix well. e. Streak for isolation using a loopful of rehydrated suspension on duplicate trypticase soy agar (TSA) or nutrient agar (NA) plates. f. Incubate broth culture (TUBE A) and plate cultures at 30±1°C for

	24±2 h. g. Record all manipulations on the Organism Culture Tracking Form (see section 14).
12.2 Culture Identification	a. Perform initial confirmation testing for quality control (QC) using the 24±2 h NA or TSA plates. b. Following the incubation period (as stated in section 12.1f), record the observed colony morphology on the NA or TSA plates and Gram stain reaction. See section 12.2d. for details on colony morphology and Gram stain reaction. c. Perform a Gram stain from growth taken from the TSA or NA plates. Perform the Gram stain according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion). d. *B. subtilis* is a Gram positive rod; colonies on TSA are opaque, rough, dull, round, with irregular margins, and low convex. Colonial variation may be observed and is typical for this strain. e. Perform VITEK™ analysis according to the manufacturer's instructions. f. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).
12.3 Generation of Stock Cultures	a. Use the 24±2 h TUBE A (see Attachment 1) broth culture discussed in section 12.1d to initiate stock cultures – streak a minimum of six NA slants with *B. subtilis* and incubate at 36±1ºC for 24±2 h. b. Following incubation, store the cultures at 2-5ºC for 30±2 days. These cultures are identified as the "stock cultures." Begin stock culture transfers as outlined in section 12.1e. Repeat the cycle for a maximum of one year. c. From a set of six stock cultures, one is used every 30±2 days for QC and to generate new stock cultures, four may be used per month (one/week) for generation of test cultures, and one is a back-up tube.
12.4 Monthly QC of Stock Cultures	a. Perform monthly QC of stock cultures just prior to or concurrently with stock culture transfers. Use one refrigerated stock culture tube and streak a loopful on a plate of TSA.

	b.	Incubate the plates at 36±1ºC for 24±2 h (18-24 h for use in the VITEK 2 Compact). Follow steps outlined in section 12.2b to confirm the identity of the organism.
12.5 Culture Maintenance	a.	Every 30±2 days inoculate a new set of stock culture tubes from a current stock culture tube. Use the same refrigerated stock culture tube used for Monthly QC described in section 12.4a to inoculate 6 new stock cultures tubes as outlined in section 12.3a.
	b.	Incubate the new stock cultures as indicated in section 12.3a.
	c.	Following the incubation period, store the stock cultures at 2-5ºC for 30±2 days.
12.6 Production of *B. subtilis* Spore Suspension	a.	Use growth from a stock culture tube to inoculate 10 mL tubes (e.g., 2 tubes, depending on the amount of spore preparation desired) of NB and incubate tubes on an orbital shaker for 24±2 h at approximately 150 rpm at 36±1ºC. Use this culture to inoculate amended NA plates. Inoculate each plate with 500 µL of broth culture and spread the inoculum with a sterile bent glass rod or suitable spreading device. In addition, verify the purity of this culture by streak isolating on amended NA (incubate at 36±1°C for 24±2 h). Wrap each plate with parafilm or place in plastic bags. Incubate plates inverted for 12-14 days at 36±1ºC.
	b.	Following incubation, harvest the spores by adding 10 mL chilled sterile water to each plate. Using a spreader (e.g., bent glass rod), remove growth from plates and pipet suspensions into 15 mL sterile conical tubes (10 plates = 14 tubes, ~10 mL each).
	c.	Centrifuge tubes at 5,000 rpm (4,500×g) for approximately 10 min at room temperature. Remove and discard supernatant. Re-suspend pellet in each tube with 10 mL cold sterile water and centrifuge at 5,000 rpm (4,500×g) for approximately 10 min. Remove and discard supernatant. Repeat twice.
	d.	Re-suspend the pellet in each tube with 10 mL sterile water. Store the spore suspension at 2-5ºC.
	e.	Examine spore suspension with a phase contrast microscope or by staining to assess quality of the spore suspension. Examine a minimum of five fields and determine ratio of spores to vegetative cells (or sporangia). Percentage of spores versus vegetative cells should be at least 95%. Spore suspension from multiple plates can be combined and re-aliquoted into tubes for uniformity.

	f. Prior to inoculation of carriers, determine spore titer of the concentrated spore suspension by plating 100 µL aliquots of serial dilutions (e.g., 10^{-5} through 10^{-7}) using spread plating on TSA plates or another comparable validated enumeration procedure. Incubate plates for 24±2 h at 36±1°C and determine titer. i. Note: When harvested and processed, ten plates of amended nutrient agar should provide 80-100 mL of concentrated spore suspension (approx. 10^9 CFU/mL). Diluting the suspension prior to carrier inoculation will be necessary; a titer of 1.0×10^8 to 5.0×10^8 CFU/mL should be adequate to achieve the target carrier count.
12.7 Preparation of Porcelain Carriers	Preparation of porcelain carriers can also be found in MB-03, Screening of Polished Stainless Steel Penicylinders, Porcelain Penicylinders, and Glass Slide Carriers Used in Disinfectant Efficacy Testing. a. Prior to use, examine porcelain carriers individually and discard those with scratches, nicks, spurs, or discolorations. b. Rinse unused carriers gently in water three times to remove loose material and drain. c. Place rinsed carriers into Petri dishes matted with 2 layers of filter paper in groups of 15 carriers per Petri dish or place carriers into 25×150 mm tubes (10 carriers per tube). d. Sterilize 20 min at 121°C. Cool and store at room temperature. Note: Handle porcelain carriers with care when placing in Petri dishes. Minimize carrier movement and avoid excessive contact between carriers that might result in chips and cracks. Wash carriers with Triton X-100 and rinse with water 4 times for reuse.
12.8 Inoculation of Porcelain Carriers	a. Dilute the concentrated spore suspension as necessary with sterile water to achieve carrier counts between 1.0×10^5 and approximately 1.0×10^6 spores/carrier. Dispense 10 mL diluted spore suspension into an appropriate number of 25×150 mm tubes. b. Add 10 sterile carriers to each tube containing 10 mL spore suspension, slightly agitate, and let stand 10-15 min. c. Remove each carrier with sterile hook and place upright in a sterile Petri dish lined with two sheets of filter paper, no more than 30 carriers per Petri dish. d. Air dry in biological safety cabinet for approximately 30±2 min. Place Petri dishes containing inoculated carriers in vacuum

	desiccator (with gauge) containing $CaCl_2$ and draw vacuum of 27" (69 cm) Hg. e. Dry carriers under vacuum for 24±2 h before use in HCl resistance testing, efficacy testing or carrier counts. Maintain in a sealed desiccation unit under vacuum (27" Hg) for up to three months. i. Inoculated carriers may be used after three months (within one year) if they meet the acceptable HCl resistance and carrier count criteria. Sterilize and reuse if necessary.
12.9 Spore Enumeration (carrier counts)	a. Prior to use, determine the carrier counts for each preparation of inoculated carriers. Assay 3 to 5 randomly selected carriers per preparation. b. Place each inoculated carrier into a 50 mL plastic, polypropylene conical centrifuge tube containing 10 mL of sterile water. c. Sonicate carriers for 5 min ± 30 s. Note: For sonication, place tubes into an appropriately sized glass beaker with tap water to the level of sterile water in the tubes. Place beaker in sonicator so that water level in the beaker is even with water level fill line on sonicator tank. Fill tank with tap water to water level fill line. Suspend beaker in sonicator tank so it does not touch bottom of tank and so all three water levels (inside test tubes, inside beaker, and sonicator tank) are the same. d. Following sonication, vortex tubes for 2 min ± 5 s. e. Dilute spore suspensions to 10^{-3} by transferring 1 mL aliquots to tubes containing 9 mL sterile water. i. Alternatively, pool the water from the tubes with the carriers and briefly vortex. Serially dilute and plate appropriate aliquots of the pooled water (30-50 mL) and calculate the average carrier count per set. f. Plate 100 µL of the 10^{0} (tube with the carrier) through the 10^{-3} dilution in duplicate using spread plating with TSA. Invert plates and incubate for 24-48 h at 36±1ºC. i. Alternatively, use 3M™ Petrifilm™ AC Plates for enumeration of the test organism. Dilute the spore suspensions through 10^{-4} and plate 1 mL aliquots on the Petrifilm. Note: Conduct a culture purity check on one dilution of

	one carrier. g. Count colonies. Record all counts less than 300 and use those counts for enumeration. Report plates with colony counts over 300 as TNTC (Too Numerous to Count). Average spore counts per carrier should be between 1.0×10^5 and approximately 1.0×10^6 spores/carrier. Do not use carriers with counts outside this range. Average spore counts per carrier should be within one log of each other.
12.10 HCl Resistance	a. Equilibrate water bath to 20±1ºC. Pipet 10 mL of 2.5M HCl into two 25×100 mm tubes, place into water bath, and allow to equilibrate. Start timer and rapidly transfer 4 inoculated penicylinders into a tube with 2.5 M HCl using flamed hooks or forceps. Do not allow carriers or transfer device to contact inside of wall of acid tube. b. Transfer individual carriers after 2, 5, 10, and 20 minutes of HCl exposure to a separate tube of modified FTM. Rotate each tube vigorously by hand for approximately 20 s and then transfer carrier to a second tube of modified FTM. c. For viability control, place one unexposed inoculated carrier in a separate tube of modified FTM. For media sterility, use one tube of modified FTM. d. Incubate all test and control tubes for 21 days at 36±1ºC. Record results as growth (+) or no growth (0) at each time period. Spores should resist HCl (i.e., remain viable) for ≥2 minutes to be qualified as resistant test spores. Discard carriers if not resistant (i.e., inactivated) and repeat inoculation of carriers as previously described.
12.11 Efficacy Test	a. Prepare disinfectant samples according to MB-22. For a 60-carrier test, place 10 mL product at dilution recommended for use or under investigation into each of twelve 25×150 mm or 25×100 mm test tubes, or use appropriate number of tubes assuming 5 test carriers per tube of test chemical. b. Place tubes in 20±1ºC water bath and let equilibrate to temperature. Using a sterile hook (or forceps), transfer inoculated carriers sequentially at 2 minute intervals in groups of 5 from Petri dish to test tubes containing sporicidal agent. Use a certified timer to monitor time. i. Flame hook and allow cooling after each transfer. When lowering carriers into test tube, neither carriers nor wire

	hook may touch sides of tubes. ii. If interior sides are touched, note tube number – do not count carrier set if any carrier from that group of 5 yields a positive result. Testing another set of five carriers is recommended. iii. Deposit carriers into test tubes within ± 5 s of the prescribed drop time. Return tubes to water bath immediately after adding carriers. c. After contact period has been achieved, transfer carriers in same sequential timed fashion into primary subculture tubes containing appropriate neutralizer (10 mL in 20×150 mm test tubes). i. Remove the carriers one at a time from the test tube with sterile hook, tap against interior side of tube to remove excess sporicidal agent, and transfer into neutralizer tube (primary tube). ii. All five carriers must be transferred during each 2-minute interval. Flame hook between each carrier transfer. Move remaining carriers into their corresponding neutralizer tubes at appropriate time. iii. Carriers may touch interior sides of neutralizer tube during transfer, but contact should be minimized. d. After each carrier is deposited, recap neutralizer tube and gently shake to facilitate adequate mixing and efficient neutralization. e. Within one hour from when last carrier was deposited into primaries, transfer carriers using sterile wire hook to second subculture tube (secondary tube) containing 10 mL of appropriate recovery medium, one carrier per tube. i. Move carriers in order, but movements do not have to be timed. Gently shake entire rack of secondary tubes after all carriers have been transferred. f. Incubate primary (neutralizer) and secondary subculture tubes for 21 days at 36±1°C. Report results as growth (+) or no growth (0). i. A positive result is one in which medium appears turbid. A negative result is one in which medium appears clear. Shake each tube prior to recording results to determine presence or absence of growth/turbidity. ii. Primary and secondary subculture tubes for each carrier

	represent a "carrier set." A positive result in either primary or secondary subculture tube is considered a positive result for the carrier set. g. Media sterility controls and system controls (check for aseptic technique during carrier transfer process) are recommended. i. For media controls, incubate 1-3 unopened subculture medium tubes with the test sample tubes for 21 days at 36±1ºC. ii. For system controls, use sterile forceps or hooks to transfer 3 sterile carriers into a tube of test chemical. iii. Transfer system control carriers to neutralizer medium as follows: at start of sample test (prior to first tube), transfer 1 sterile carrier to tube of neutralizer medium. After one half of test carriers have been transferred to neutralizer tubes, transfer a second sterile carrier to tube of neutralizer medium. After all test carriers (last tube) have been transferred to neutralizer tubes, transfer third sterile carrier to tube of neutralizer medium. iv. Transfer system control carriers to secondary subculture medium as follows: immediately prior to initiating transfer of test carriers into secondary subculture medium tubes, transfer first system control sterile carrier from neutralizer medium to tube of subculture medium. After one half of test carriers have been transferred to secondary subculture medium tubes, transfer second system control sterile carrier to tube of subculture medium. After all test carriers have been transferred to secondary subculture medium tubes, transfer third system control sterile carrier to tube of subculture medium. v. For each test, include a positive carrier control by placing one inoculated carrier into tube of secondary subculture medium. Incubate controls and test sample tubes together for 21 days at 36±1ºC. h. Perform presumptive identification on a minimum of three positive carrier sets per test, if available, using Gram stain and/or plating on TSA. Additional confirmation may be performed using VITEK or comparable method. i. If there are fewer than three positive carrier sets, confirm growth from each positive carrier set. If both tubes are

	positive in carrier set, select only one tube for confirmatory testing. For tests with 20 or more positive carrier sets, confirm at least 20% by Gram stain. If Gram stains are performed from growth taken directly from positive tubes, the staining should be performed within 5-7 days of conducting the efficacy test. See section 12.2d. for Gram stain reaction and colony characteristics.
12.12 Neutralization Confirmation Procedure	a. Perform a neutralization confirmation test in advance or in conjunction with efficacy testing. This assay is designed to simulate the conditions (i.e., neutralizer, subculture medium, contact time, diluent, concentration of test substance) of the efficacy test and to demonstrate the recovery of a low level of spores (e.g., 5-100). Diluted inoculum (e.g., spores of *B. subtilis*) is added directly to the various sets of subculture media tubes (see Table 1). This assay provides for a quantitative approach to assessing the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer itself or neutralizer-disinfectant interactions. b. Produce a spore preparation according to the procedure for amended nutrient agar. Harvest growth from plates (e.g., five plates) per the method, except re-suspend pellet after final centrifugation step in approximately 100 mL aqueous (40%) ethanol. i. Determine spore count by serial dilution and plating on TSA. Desirable target of the initial working suspension is 1.0×10^{8} to 1.0×10^{9} CFU/mL. The suspension may require adjustment to reach target titer. ii. Prepare serial ten-fold dilutions of the inoculum in sterile water out to 10^{-7}. Use 100 µL aliquots of the 10^{-5}, 10^{-6} and 10^{-7} dilutions to inoculate the neutralizer and subculture media tubes – the target number of spores to be delivered per tube in this assay is 5-100 per tube. iii. Determine spore titer by plating each of three dilutions in duplicate on TSA agar. Incubate plates inverted for 24-48 h at 36±1ºC. Count colonies. Report plates with colony counts over 300 as TNTC. Note: A standardized spore preparation adjusted to deliver 5-100 spores/mL may be substituted for the three dilutions of spore inoculum. In addition, spores sheared from

inoculated carriers may be used as a working suspension.

c. Use 5 sterile porcelain carriers (only 3 to be used in the assay). Within 5 s, place a set of 5 carriers into a test tube (25×150 mm or 25×100 mm) containing test chemical; transfer carriers according to section 12.11b. Allow carriers to remain in test chemical per the specified contact time and temperature.

   i. After the contact time is complete, aseptically transfer three of the five carriers individually into tubes containing the neutralizer per section 12.11c. This set of tubes is the Neutralizer/Primary Subculture treatment.

   ii. Following the transfer of the last carrier into neutralizer tube, transfer each carrier, in sequence, into tube containing secondary subculture medium. This portion of assay is not timed, but should be made as soon as possible. This set is the Secondary Subculture treatment.

d. Following carrier transfer, inoculate each tube (Neutralizer/ Primary and Secondary Subculture treatment tubes) with 100 µL of each of three inoculum dilutions (10^{-5}, 10^{-6} and 10^{-7}).

e. For controls, use three fresh unexposed tubes of neutralizer and three tubes of the secondary subculture medium; also inoculate each control tube with 100 µL of each of three inoculum dilutions. Include one uninoculated tube of neutralizer and secondary subculture media to serve as sterility controls.

f. See Table 1 for tube inoculation scheme.

g. Incubate all tubes 5-7 days at 36 ± 1ºC.

h. Record results as growth (+) or no growth (0). The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of inoculum (spores) is added to the subculture tubes.

Table 1. Neutralization confirmation procedure – inoculating treatment and control tubes with diluted spore suspension*

Treatment	Dilution & Tube #
Neutralizer-Primary Subculture Treatment	100 µL of 10^{-5} → Tube 1 100 µL of 10^{-6} → Tube 2 100 µL of 10^{-7} → Tube 3
Secondary Subculture Treatment (with Carrier)	100 µL of 10^{-5} → Tube 1 100 µL of 10^{-6} → Tube 2 100 µL of 10^{-7} → Tube 3
Neutralizer-Primary Inoculated Control	100 µL of 10^{-5} → Tube 1 100 µL of 10^{-6} → Tube 2 100 µL of 10^{-7} → Tube 3
Secondary Subculture Inoculated Control	100 µL of 10^{-5} → Tube 1 100 µL of 10^{-6} → Tube 2 100 µL of 10^{-7} → Tube 3

*Use of 10^{-5} through 10^{-7} based on an approx. starting suspension of 10^{8} spores/mL

i. Confirm a minimum of one positive per treatment and control (if available) using Gram staining and colony morphology on TSA, see section 12.2d. For each treatment and control group, conduct confirmation testing on growth from tube with fewest spores delivered.

j. Growth in the inoculated controls verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes.

k. The occurrence of growth in the Neutralizer/Primary Subculture and Secondary Subculture treatment tubes is used to assess the effectiveness of the neutralizer. No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions.

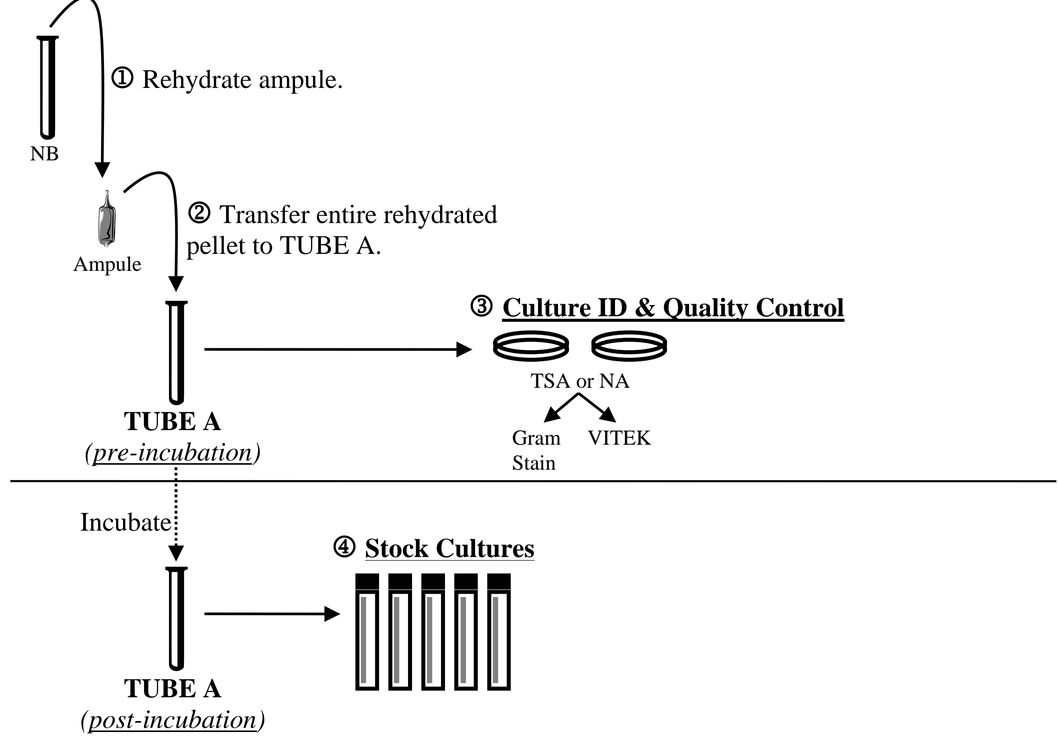
l. For a neutralizer to be deemed effective, growth must occur in the Secondary Subculture treatment tubes which received lower levels of inoculum (e.g., 5-100 CFU/mL).

m. Growth in the Secondary Subculture Inoculated Control verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. No growth or only growth in tubes

	which received high levels of inoculum (e.g., a dilution with plate counts which are too numerous to count) indicates poor media performance. n. Growth in the Neutralizer-Primary Inoculated Control should be comparable to the Secondary Subculture Inoculated Control if the neutralizer is the same as the secondary subculture media. There may be cases when the neutralizer is significantly different from the secondary subculture media. In these cases, growth may not be comparable to the Secondary Subculture Inoculated Control. o. The Neutralizer-Primary and Secondary Subculture Uninoculated Control tubes are used to determine sterility and must show no growth for the test to be valid.
13. Data Analysis/ Calculations	1. Data will be recorded on data sheets (see section 14). Calculations will be computed using a Microsoft Excel spreadsheet (see section 14). Electronic copies of the spreadsheet as well as hard copies will be retained. 2. To calculate CFU/mL per carrier: $\frac{(avg.\ CFU\ \ for\, 10^{-w})+(avg.\ CFU\ \ for\, 10^{-x})+(avg.\ CFU\ \ for\, 10^{-y})+(avg.\ CFU\ \ for\, 10^{-z})}{10^{-w}+10^{-x}+10^{-y}+10^{-z}}$ where 10^{-w}, 10^{-x}, 10^{-y}, and 10^{-z} are the dilutions plated. In the event that one or more dilutions yield plate counts greater than 300, those counts and their corresponding dilutions will not be used in the calculations. If only one of two plates has counts yielding 300 CFU or less, forgo its use in calculations in place of counts from a subsequent dilution; if none exists, use only the countable plate for calculations (e.g., do not average the TNTC (as 300) and the countable plate). Note: Include plate counts of 0 in all calculations. 3. To calculate CFU/carrier, multiply the CFU/mL per carrier by the volume of media used to suspend carrier for sonication or vortexing. Round numbers and use only two significant figures to calculate averages and CFU/carrier. 4. Calculate the average CFU/carrier for all carriers tested.
14. Forms and Data Sheets	1. Attachment 1: Culture Initiation and Stock Culture Generation Flow Chart for *B. subtilis* 2. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record MB-03_F1.docx

	SAT: Organism Culture Tracking Form	MB-15-04_F1.docx
	SAT: Test Microbe Confirmation Sheet (Quality Control)	MB-15-04_F2.docx
	SAT: Carrier Enumeration Form	MB-15-04_F3.docx
	SAT: Carrier Count Spreadsheet	MB-15-04_F4.xlsx
	SAT: HCl Resistance Test Data Sheet	MB-15-04_F5.docx
	SAT: Information Sheet	MB-15-04_F6.docx
	SAT: Time Recording Sheet for Carrier Transfers	MB-15-04_F7.docx
	SAT: Results Form (1-30)	MB-15-04_F8.docx
	SAT: Results Form (31-60)	MB-15-04_F9.docx
	SAT: Performance Controls Results Sheet	MB-15-04_F10.docx
	SAT: Test Microbe Confirmation Sheet	MB-15-04_F11.docx
	SAT: Neutralization Confirmation Assay Information Sheet	MB-15-04_F12.docx
	SAT: Neutralization Confirmation Assay Results Form	MB-15-04_F13.docx
	SAT: Neutralization Confirmation Assay Time Recording Sheet for Carrier Transfers	MB-15-04_F14.docx
	SAT: Neutralization Confirmation Assay Serial Dilution/Plating Tracking Form	MB-15-04_F15.docx
	SAT: Neutralization Confirmation Assay Inoculum Enumeration Form	MB-15-04_F16.docx
15. References	1. Official Methods of Analysis (Revised 2013) 21st ED., AOAC INTERNATIONAL, Method 966.04, Gaithersburg, MD, Chapter 6 2. Standard Methods for the Examination of Water and Wastewater. 23rd Ed. American Public Health Association, 1015 15th Street, NW, Washington, DC 3. Tomasino, S.F. & Hamilton, M.A. (2006) *JAOAC* Int. 89, 1373-1397	

Attachment 1: Culture Initiation and Stock Culture Generation Flow Chart for *B. subtilis*



a. Obtain lyophilized cultures annually from ATCC. Using a tube containing 5-6 mL of NB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the pellet for *B. subtilis.*

b. Aseptically transfer the entire rehydrated pellet back into the original tube of nutrient broth designated as "TUBE A." Mix well. Use suspension in TUBE A for CULTURE ID & QUALITY CONTROL. Incubate TUBE A for *B. subtilis* for 24 h at 30±1°C.

c. Culture ID and Quality Control. Using a loopful of rehydrated suspension from TUBE A, streak for isolation on duplicate plates (NA or TSA). Incubate plates at 30±1°C for 24 h. Record results on the Test Microbe Confirmation Sheet.

d. Stock Culture Generation. Using the 24±2 h TUBE A broth culture: initiate stock cultures by streak-inoculating six NA slants. Incubate the slants at 36±1ºC for 24±2 h. Record all manipulations on the Organism Culture Tracking Form.

Reference #7

FDA Submission – Bottle Closure System
Leak test report

Dye Penetration Study

Spray Nozzle of an 8 Ounce PET Bottle

REPORT NO. AVL-1065-R

Prepared by:

Name: Pablo Fernandez: ______________ Date: 11/21/2022
Quality Assurance Manager

Approved by:

Hoji Alimi – CEO ______________ Date: 11/21/2022
Research & Development

Monica Shaffer – Manager: ______________ Date: 11/21/2022
Manufacturing

Stephanie Alimi - Manager: ______________ Date: 11/21/2022
Operations

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1. INTRODUCTION:

This report has been prepared to detail the results obtained from conducting the experiments and the mentioned studies in protocol AVL-1065-P (Dye Penetration Study: Spray Nozzle of an 8-ounce PET Bottle).

2. TEST ARTICLES:

Four 8-ounce PET bottles and finger pump spray heads were released by Quality Assurance for the purposes of this testing evaluation, the bottles were filled with DI water as instructed per protocol AV-1065-P:

- 3 - 8-ounce bottles were filled with DI water and used for Dye Penetration Test.
- 1 – 8-ounce bottle was filled with DI water as "Control".

The three samples were then immersed in dye solution and tested for potential back absorption of liquid post operation of the finger spray. Each container/closure was operated per Protocol AV-1065-P, by spraying 120 times. This would allow for approximately less than 7% of the volume to be sprayed out from each bottle.

The 3 bottles were then retrieved, wiped and cleaned per Protocol AV-1065-P and allowed to air dry in a horizontal hood. The content of each bottle, including control bottle was then examined by Quality Assurance, photographed and included herein as a reference.

3. TEST RESULTS:

The results of this study have demonstrated that Spectricept PET bottle and finer spray allow for air (solution under the test conditions) to replace the volume of solution sprayed out. The results of this study clearly demonstrates that when Spectricept bottler closure is used in a non-sterile environment, it can introduce/force potential contamination from the outside into the bottle closure.

TABLE 1 – BOTTLE / CLOSURE INFORMATION

Sample Control	Liquid	Bottle Type	Closure Type	MS Number	Lot #
Sample #1	Clear DI Water	8oz PET White Boston Round	Fine Mist Sprayer, 24-410	Bottle MS100-06 Closure MS100-03	Lot# 20201020-01 Lot# 20200915-10
Sample #2	Clear DI Water	8oz PET White Boston Round	Fine Mist Sprayer, 24-410	Bottle MS100-06 Closure MS100-03	Lot# 20201020-01 Lot# 20200915-10
Sample #3	Clear DI Water	8oz PET White Boston Round	Fine Mist Sprayer, 24-410	Bottle MS100-06 Closure MS100-03	Lot# 20201020-01 Lot# 20200915-10
CONTROL	Clear DI Water	8oz PET White Boston Round	Fine Mist Sprayer, 24-410	Bottle MS100-06 Closure MS100-03	Lot# 20201020-01 Lot# 20200915-10

FINAL RESULTS:

The following are the results of testing evaluation as further detailed in Protocol No. AV-1065-P. The following results supports the use of HOCl as a preservative to protect the integrity of the solution when used in wound care. This information complies with the predicate device Microcyn Wound and Skin as cleared by FDA.

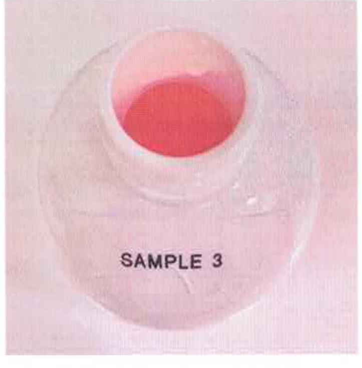
TABLE 2 – COLOR RESULTS

Sample Control	Pre- Test Content Color	Number of Sprays Operated	Post- Test Liquid Color
Sample #1	Clear	120 pumps	Red
Sample #2	Clear	120 pumps	Red
Sample #3	Clear	120 pumps	Red
CONTROL	Clear	N/A	Clear – No Dye

Sample Bottle #1

Sample Bottle #2

Sample Bottle # 3



Control Bottle

Sample Closure #1

Sample Closure #2

Sample Closure #3

Control Closure

Reference #8

In dependent laboratory - Time Kill Study
Per USP Guidelines

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Final Report number: 2020-01

Time Kill Study Per USP Guidelines

Test Article: Spectricept B (F-474)

Report Date: March 17, 2020

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Date 3/17/2020

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Date 17 Mar 2020

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Table of Contents

Purpose :

Spectricept B (F-474) was provided as finished final packaged product intended for efficacy testing against two microorganisms (Methicillin-resistant *Staphylococcus aureus* (MRSA) USA 300 and *Pseudomonas aeruginosa ATCC 27853*) and HIV virus.

References :

Time kill study was conducted in compliance with ASTM E2315-16, Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure, ASTM International, West Conshohocken, PA, 2016, www.astm.org

ASTM 1053-11 for Viral testing

Test Method Procedure:

Bacterial Time Kill Study:
Test article (F-474) was received and stored at room temperature and away from any direct light. The product was tested against both Methicillin-resistant Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa. Each test required the test formulation to be challenged for 20 minutes in presence of blood serum (Fetal Bovine Serum). Upon completion of 20 minutes exposure time, the suspension containing the test product, serum and microorganisms were subject to a serial dilution in DE broth. Each tube was vortexed and 100 microliter from each test tube was pipetted onto TSA plate. All TSA plates were incubated at 30C-35C for 48 hours +/- 2hours. The data was enumerated and used to determine log reduction in comparison to positive control. Log reduction calculations were accomplished by Collidion visiting scientist.

Appropriate positive control, negative controls were prepared and incubated for incubation as described above and enumerated.

Anti-viral study:

Test article was received and stored at room temperature and away from any direct light. The product was tested against HIV-1 (NL4.3) to determine its antiviral efficacy. The viral suspension was prepared in 5% serum (Fetal Bovine Serum) . The viral suspension along with 5% serum was pipetted onto a sterile plastic petri plate and allowed to air dry for no more than 10 minutes within a biological safety cabinet. The product was then introduced onto the viral film for 10 minutes at room temperature. A cell suspension of human T cell leukemia - C8166 was added to the viral film/product. The cell lines were then incubated at 37°C for 48 hours +/- 2 hours. After incubation, an Elisa test was conducted using 100ul of supernatant for P24 protein assay. The attached interim report includes detailed procedure and results using the p24 Elisa assay.

The infected cell lines will continue to incubate for 8 days to conduct the CPE (cytopathic assay) The CPE report is pending for HIV-1.

Conclusion:

Spectricept B (F-474) demonstrated excellent antimicrobial activity against the two bacterial strains and one viral strain.

The test results have shown that Spectricept (F-474) is capable of providing ≥7.21 log reduction in presence of serum against Methicillin-resistant *Staphylococcus aureus* (MRSA) USA 300 and ≥6.98 log reduction against *Pseudomonas aeruginosa ATCC 27853*.

The test results also have shown that Spectricept (F-474) has been able to inactivate HIV-1 virus after 10 minute exposure and in presence of 5% serum load.

Attachments

8708-01 Microbial Inoculum Preparation and Plating (procedure)
8708-02 Microbial Testing (Time-kill Assay) and Neutralization (procedure)
8708-02 attachment 1 (results)
8708-02 attachment 2 (results)
Testing of Disinfectant on HIV-1 (NL4.3) -infected C8166 T Cell Lines (procedure and result)

Reference #9

Peer reviewed published paper – A comparison of air sampling methods for clostridium difficile edospore aerosol

Advertisement

Original Paper | Open Access | Published: 08 February 2019

A comparison of air sampling methods for *Clostridium difficile* endospore aerosol

Casey W. Cooper, Kathleen A. N. Aithinne, Evan L. Floyd, Bradley S. Stevenson & David L. Johnson

Aerobiologia **35**, 411–420 (2019) | Cite this article

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Abstract

The airborne dissemination of *Clostridium difficile* (*C. difficile*) endospores (spores) in healthcare environments is documented in multiple studies. Once airborne, spores have the potential for transport on air currents to other areas. This study compared the methods in the collection of *C. difficile* spore aerosol. This study determined the relative efficiency of commonly used bioaerosol air sampling methods when characterizing airborne *C. difficile* spore concentrations. Air samplers evaluated in this study were the AirTrace slit-to-agar impactor, AGI-30 impinger, SKC BioSampler impinger, and a 47-mm mixed cellulose ester (MCE) filter cassette. Non-toxigenic *C. difficile* spores were nebulized into an enclosure contained in a biological safety cabinet. Side-by-side air samples were drawn from the enclosure. The slit-to-agar impactor, successfully used in previous studies to collect airborne spores, served as the reference method. Relative efficiency for the 47-mm MCE filter cartridge was higher than the slit-to-agar impactor (mean 136.6%, 95% CI 124.7–148.5%). Efficiencies of the impingers were similar and were low (mean 4.13%, 95% CI 2.27–5.99%). Impingers failed to maintain culturability of *C. difficile* spores during sampling. This study is the first to compare the efficiencies of commonly used bioaerosol sampling methods to collect airborne *C. difficile* spores. Filter air sampling provided the greatest collection of airborne spores. Slit-to-agar air sampling may underestimate the number of airborne spores present. Impinger air sampling could significantly underestimate the actual number of airborne *C. difficile* spores present or fail to detect airborne spores.

1 Introduction

1.1 Background

Healthcare-acquired *Clostridium difficile* infection (CDI) is a significant burden to US healthcare facilities and their patients, with an estimated 453,000 incident CDI and 29,000 deaths in 2011 (Lessa et al. 2015). Of these nearly half million cases, an estimated 293,300 were healthcare-associated with 107,600 hospital onset, 104,400 nursing home onset, and 81,300 post-outpatient care onset. CDI is the leading cause of gastroenteritis-associated death, causing 14,000 deaths in 2007 alone (Hall et al. 2012), was responsible for an estimated $4.8 billion in excess healthcare costs for acute care facilities in 2008 (Dubberke and Olsen 2012), and has become one of the most common healthcare-acquired infections in the USA (Magill et al. 2011).

CDI patients experience multiple episodes of watery diarrhea each day, and both *C. difficile* bacterial endospores (spores) and vegetative cells are shed in stool. Spore concentrations in

symptomatic CDI patients may be from 10^4 to 10^6 spores per gram of stool (Kim et al. 1981; Naaber et al. 2011), and vegetative cell concentrations may be an order of magnitude more abundant (Jump et al. 2007). Whereas vegetative cells die off rapidly in the environment on dry surfaces (Jump et al. 2007), spores are resistant to environmental degradation and will survive in the environment for long periods of time (Gerding et al. 2008). The spores are infectious, and CDI transmission is believed to be primarily via hand contact with the patient and contaminated environmental surfaces during patient care (McDonald et al. 2018).

Environmental contamination of CDI patient care rooms and adjacent areas with *C. difficile* spores is a recognized contact transmission risk factor, which has been demonstrated in numerous studies conducted since the 1980s (Kim et al. 1981; McDonald et al. 2018). Culturable spores have been collected from surfaces in treatment rooms with symptomatic patients, asymptomatic patients, and even patients with no evidence of *C. difficile* colonization. Recent clinical practice guidelines for CDI prevention and control include: accommodate CDI patients in a private room with a dedicated toilet if possible, or cohort CDI patients if insufficient rooms are available; gown and glove upon entry to a CDI patient room and during patient care; perform hand hygiene before and after CDI patient contact; continue precautions for at least 48 h after diarrhea has resolved; perform terminal room cleaning with a sporicidal agent; and consider daily cleaning with a sporicidal agent during outbreaks or in hyper-endemic settings (McDonald et al. 2018). Because CDI is only considered a contact transmission risk, negative pressure isolation in an engineered Airborne Infection Isolation Room is not included.

These practice guidelines implicitly assume that spore contact risk is limited to the patient, fomites in the patient room, and room surfaces; however, several studies have shown that *C. difficile* spores are intermittently aerosolized during patient care activities such as bedding changes, toilet flushing, and patient feeding or meal delivery. These spores can remain airborne for extended periods (Aithinne et al. 2018; Best et al. 2010, 2012; Roberts et al. 2008). These "droplet nuclei" spore aerosols could then travel with air currents and may contaminate environmental surfaces remote from the patient room. This airborne transport and deposition may provide additional, and likely unsuspected, opportunities for contact transmission. However, few studies have been successful in isolating *C. difficile* from air samples in healthcare environments (Roberts et al. 2008; Best et al. 2010, 2012).

Commonly used bioaerosol sampling methods include liquid impingement (e.g., SKC BioSampler and AGI-30 impingers), filtration (e.g., mixed cellulose ester and gelatin filters), and direct-to-agar impaction (e.g., MB2 and N6 multi-hole impactors, AirTrace slit-to-agar impactor) (Willeke and Macher 1999). Filtration is the simplest of the air sampling methods. A filter with support pad is placed in a cassette that is connected to a calibrated air sampling pump. The air flow rate may be selected from within a range of allowable values determined by the filter media and filter area. Filters may be extracted into a liquid for subsequent spread plating on agar-filled culture dishes, or placed directly on the agar surface. Gelatin filters will dissolve into the agar, whereas mixed cellulose ester (MCE) filters must absorb agar media in sufficient amounts to support colony development. Liquid impingers direct a high-velocity jet of particle-laden air onto the surface of collection fluid. The jet is forced to change directions abruptly, and the particles' inertia causes them to strike the liquid and be captured. Whereas the AGI-30 has a single nozzle oriented normally to the liquid surface, the BioSampler has three nozzles oriented at an angle to the liquid surface. The angled nozzles are intended to reduce stress on captured organisms and minimize re-aerosolization of captured organisms from the collection fluid. After sampling, an aliquot of the collection fluid (diluted as necessary) may be spread onto agar-filled culture dishes; alternatively, the fluid may be filtered and the filter was then placed on agar for incubation. The slit-to-agar impactor directs a high-velocity stream of air through a narrow slit and impinges it onto the surface of an agar-filled culture dish, much like a knife preparing to cut into the radius of a birthday cake. The sheet of air must make an abrupt 90° turn, causing entrained particles to impact on the agar due to their inertia. The agar plate is slowly rotated under the inlet slit, so that particles will

deposit over the agar surface area covered by the moving line of impingement. Collection plates from jet-to-agar or slit-to-agar impactors are directly incubated. To date, no studies have been reported that compared the relative performance of these methods when sampling for airborne *C. difficile* spore aerosol, and only one of these (slit-to-agar impaction) has been successful in detecting airborne *C. difficile* in a healthcare environment (Best et al. 2010).

Characterization of airborne *C. difficile* spore concentrations is needed to fully perform risk assessments for the transport of *C. difficile* in healthcare environments. It is therefore of interest to determine which sampling methods are most efficient for determining *C. difficile* spore aerosol presence in healthcare environments so that strategies can be developed for quantifying spore aerosol generation sources and rates during patient care, characterizing spore migration patterns in the care environment, and developing more effective strategies for minimizing transmission risk from patient contact with aerosol-transported spores.

1.2 Goals of this investigation

Our goal was to determine the relative efficiency of inexpensive and commonly used bioaerosol air sampling methods compared to the more expensive (but known to be successful) slit-to-agar method when characterizing airborne *C. difficile* spore concentrations. We compared, under controlled conditions, the relative capture efficiencies of commonly used collection devices based on liquid impingement, slit-to-agar impaction, and filtration. Our hypothesis was that the sampling efficiencies of filter- and impinger-based sampling methods would be comparable to those of the more expensive slit-to-agar method successfully used in previous studies.

2 Methods

2.1 Spore suspensions

All experiments were performed using a non-toxigenic strain of *C. difficile* (ATCC 700057, Microbiologics, St Cloud, MN). Spore suspensions were prepared after the method of Aithinne et al. (2018). Briefly, source organisms were placed in 500 mL of brain–heart broth and incubated anaerobically at 37 °C for 10 days. This extended incubation time ensured depletion of broth nutrients, resulting in sporulation. The resulting spore suspension was heat-shocked at 80 °C for 20 min to remove any remaining vegetative cells. The spore suspension was then mixed and separated into 50-mL aliquots to be centrifuged for 15 min at 5000*g*. For each aliquot, supernatant broth was decanted, and the pelleted spores were re-suspended in sterilized water and were pelleted again via centrifugation for 15 min at 5000 g. The washing step was repeated three more times, and the final suspensions were refrigerated at 4 °C until needed.

2.2 Air samplers

The bioaerosol samplers evaluated were the AGI-30 liquid impinger (Ace Glass, Vineland, NJ), BioSampler liquid impinger (SKC, Eighty-Four, PA), AirTrace rotating plate slit-to-agar impactor (Particle Measuring Systems, Boulder, CO), and mixed cellulose ester (MCE) filters in conductive cassettes (MilliporeSigma, Burlington, MA) (Fig. 1). As previously noted, the liquid impingement and filtration-based devices are commonly used for bioaerosol sampling (Willeke 1999). MCE filters were included both because they are commonly used and because Xu et al. (2013) found that airborne bacteria in indoor environments could be cultured from MCE filters placed directly on ChromAgar® media. MCE filters were also shown to be compatible with ChromAgar media for enumerating *C. difficile* spores in water when placed directly onto the media surface (Aithinne et al. 2018). Gelatin filters were excluded due to their tendency to dry out during sampling (Macher and First 1984) and because its potential effect on *C. difficile* culturing on ChromAgar® was unknown. The rotating plate slit-to-agar impactor was included as the reference device because of its success in isolating airborne *C. difficile* in recent studies (Best et al. 2010, 2012; Aithinne et al. 2018).

Fig. 1

Air samplers used in the study included (left–right) the AGI-30 impinger, BioSampler impinger, 47-mm MCE filters in conductive cassettes, and AirTrace slit-to-agar rotating plate impactor (not to scale)

Full size image

Low suspension concentrations were expected, so in this work we chose to filter the undiluted impinger liquid to maximize our detection limit and minimize potential variability due to inhomogeneous suspensions. Distilled water or phosphate-buffered saline is typically used as the collection fluid. PBS was selected as the impinger fluid due to its demonstrated effectiveness at preserving *C. difficile* spores in long-term storage up to 56 days without the loss of viability (Freeman and Wilcox 2003). The total colonies counted divided by the total volume of air sampled provides an estimate of the average air concentration during the sampling period, expressed as colony-forming units per cubic liter of air sampled (CFU/L). Both impingers were sampled at 12.5 L/min ± 10% and were filled with 20-mL sterile PBS (Sigma-Aldrich, St. Louis, MO) per manufacturer recommendations. Under these conditions, both types of impingers have been shown to have an absolute capture efficiency for particles 1 µm aerodynamic diameter of approximately 80–90% for the AGI-30 when sampling 1.1-µm *Bacillus cereus* spores (Grinshpun et al. 1997) or 1.0-µm inert particles (Willeke et al. 1998) and approximately 96% for the BioSampler sampling 1.0-µm inert particles (Willeke et al. 1998). Larger size particles up to 4 µm size have similar or higher absolute capture efficiencies for both types of impingers (Kesevan et al. 2010). The physical size of *C. difficile* spores is in the range at approximately 1–1.5 µm length and 0.5–0.7 µm diameter (Snelling et al. 2010). With a dry spore density of approximately 1.42 g/mL (Tisa et al. 1982), this results in an orientation-averaged aerodynamic diameter in the range of 1.42–1.87 µm (Johnson et al. 1987).

The AirTrace impactor was sampled at a measured air flow rate of 25.5 L/min ± 10%. Under these conditions, the impactor has a high sampling efficiency for particles of *C. difficile* spore size (Hinds 1999).

We used 45-mm-diameter, 0.45-µm pore-size MCE filters (MilliporeSigma, Burlington, MA) that allowed sampling at the same rate as the slit-to-agar impactor, i.e., 25.5 L/min. After sampling, filters were placed directly onto agar-filled culture plates for incubation and counting, with average air concentrations again expressed in CFU/L. This technique of direct placement of an MCE filter onto agar was used in the collection of environmental bacteria by Xu et al. (2013). Post-sampling, the conductive filter cartridges were decontaminated with a 10% sodium hypochlorite bleach solution, rinsed, and reused with fresh filters. To verify effective decontamination, a blank filter was incorporated in each day of trials.

Clostridium difficile-selective ChromAgar® chromogenic agar (ChromAgar, Paris, France) was used in the impactor plates and in the plates used to culture air sample filters and filters used to recover spores from impinger liquid. This agar contains antibiotics to inhibit other organisms, as well as a reagent that causes the *C. difficile* colonies to fluoresce under ultraviolet light. The media also wicks efficiently into MCE filters when they are placed on the agar surface, which is necessary for the filter culture technique. All plates were anaerobically incubated at 37 °C for 24 h, and colonies were counted under 365-nm UV illumination per manufacturer specifications.

2.3 Experimental apparatus for side-by-side air sampling

University of Oklahoma Health Sciences Center Institutional Biosafety Committee approval was obtained before conducting any experiments with *C. difficile*. Side-by-side air sampling with liquid impingers, the slit-to-agar impactor, and MCE filters was conducted in an aerosol containment chamber. A 75-L volume transparent plastic aerosol containment chamber was constructed, into which *C. difficile* spore aerosol could be generated using a 3-jet Collison MRE-type air jet nebulizer (Model CN-24, Mesa Labs, Butler, NJ). The chamber was placed within a Type II biological safety cabinet (BSC) to provide secondary containment and HEPA-filtered air for the containment chamber. Aerosol was sampled from the chamber by multiple samplers simultaneously, providing side-by-side comparisons. Due to space limitations, experiments were conducted in blocks with the slit-to-agar impactor and the two impingers as one combination, and the slit-to-agar impactor and two MCE filter cassettes as the other combination. The slit-to-agar impactor served as the "reference sampler" against which the impingers and filters were compared.

The impingers or filter cassettes could be placed inside the chamber, but due to its size the slit-to-agar impactor had to be placed outside the chamber with a 85-cm-long, 12.5-mm ID Tygon® 3606 tube conducting aerosol from the chamber to the instrument's inlet. This setup is similar to that used in previous air sampling studies by Best et al. (2012) utilizing a slit-to-agar impactor with air sample supplied by a Tygon 3606 inlet tube. The potential for losses to tubing walls and bends was assessed through penetration calculations and found to be negligible (1%) for the tubing size and flow conditions used in this work. The impingers, or the MCE filters, were placed to either side of the impactor tube inlet. A 47-mm MCE filter cassette was placed in line between the impinger exhaust and the air pump to capture any spores sampled but not captured, or captured and re-aerosolized. The impinger samplers were operated at 12.5 L/min ± 10% air flow rate and the impactor at 25.5 L/min ± 10%. The filter samplers were operated at 25.5 L/min to match the flow rate of the slit-to-agar impactor. Total air sampled from the chamber was thus approximately 53 L/min for the impinger trials and 76.5 L/min for the filter trials. The nebulizer air flow rate to the chamber was 10 L/min, so an additional 43 L/min of HEPA-filtered makeup air was drawn into the chamber from the BSC interior via relief holes in the chamber walls for the impinger trials and 66.5 L/min for the filter trials. Aerosol generated into the chamber was mixed by an 80-mm-diameter circular air fan. Uniform aerosol distribution across sampling points was verified in a series of nine trials using three MCE filter cassettes placed at different locations in the chamber, for which one-way ANOVA on these side-by-side measures showed no significant difference in indicated concentration at the three sampling locations (data not shown). Nevertheless, impinger location left or right of center for the two impinger types was alternated between impinger trials.

Four different AGI-30 samplers and three BioSamplers were used over the series of trials. The sampler flow rates were verified to be within 10% of their 12.5 L/min design flow. After each impinger trial, the impinger collection fluid was filtered through a 47-mm-diameter, 0.45-μm pore-size MCE filter, which was then placed directly onto a 65-mm-diameter ChromAgar® culture plate and anaerobically incubated at 37 °C for 24 h. The agar readily wicked into the filter matrix. Due to the low concentrations of culturable spores in the impingers, this census method provided a lower limit of detection than would be possible by surface plating a 0.1-mL aliquot. Filters from air filtering sample trials were also placed directly on ChromAgar® plates for culturing.

Sampling efficiencies were assessed relative to the slit-to-agar impactor, which has been shown to be capable of culturing *C. difficile* from healthcare environment air samples (Best et al. 2010). Air sampling time in all trials was 10 min, beyond which excessive impinger fluid losses might occur. For each trial, *C. difficile* air concentrations indicated by the two impingers or by the MCE filters were divided by the concentration indicated by the slit-to-agar impactor to provide measures of relative sampling efficiency.

2.4 Evaluation of impinger culturability retention and re-aerosolization

To evaluate the retention of culturable *C. difficile* spores by impinger air samplers, AGI-30s and BioSamplers were seeded with concentrated spore suspensions in PBS. Concentrations ranged from 500 to 1200 CFU/mL and the impingers drew HEPA-filtered, particle-free air through the sampler for 10 min at 12.5 L/min ± 10%. 0.1-ml aliquots of the impinger fluid, collected before and after the impinger operation, was spread onto a ChromAgar®-filled culture plate and anaerobically incubated for 24 h. Culturability retention was determined as a ratio of the number of culturable organisms in the impinger before and after impinger operation, as calculated from the colony counts, plated volume, and fluid volumes before and after operation.

2.5 Assessment of impinger re-aerosolization potential

We conducted an additional experiment to assess re-aerosolization, which is known to occur to some extent in liquid impingers due to fluid agitation (Grinshpun et al. 1997; Kesevan et al. 2010). We placed an MCE filter cassette in line between a BioSampler impinger and its air pump, seeded the impinger fluid with concentrated spore suspension at concentrations ranging from 500 to 1200 CFU/mL, and drew HEPA-filtered, particle-free air through the sampler for 10 min at 12.5 L/min ± 10% as before. Spore concentrations in the impinger fluid were assessed by surface plating dilutions of 0.1-mL aliquots taken immediately after seeding, and again after the 10-min air flow period. Downstream air filters were cultured on agar plates as before. Liquid volumes in the impingers were measured at initial seeding and again after impinger operation. Total culturable organisms present before and after impinger operation were calculated from the colony counts, plated volumes, and pre- and post-operation fluid volumes.

3 Results

Example of slit-to-agar and filter culture plates is shown in Fig. 2. *C. difficile* colonies were well defined and easily identified under UV illumination. No non-fluorescent colonies of other types were apparent on the plates, thus verifying the absence of contamination in the test chamber.

Fig. 2

Representative MCE filter (left) and slit-to-agar impactor plate (right)

Full size image

Nine sets of slit-to-agar versus impinger trial data and ten sets of slit-to-agar versus air filter trial data were collected. Slit-to-agar plate counts ranged from 30 to 73 (mean 49, median 49) for the slit-to-agar versus filter trials, while air filter counts ranged from 31 to 107 (mean 67, median 64). Slit-to-agar plate counts ranged from 67 to 150 (mean 108, median 106) for the slit-to-agar versus impinger trials, while impinger counts ranged from 0 to 4 for the AGI-30 and 0 to 5 for the BioSampler. Total counts for the two impingers ranged from 0 to 9 (mean 4, median 4).

Total CFU counts on the in-line exhaust filters for the two impingers ranged from 3 to 14 (median 9), which together sampled at approximately the same rate as the slit-to-agar impactor. This total was less than 10% of the culturable spores drawn into the two impingers, as estimated from corresponding slit-to-agar samples. This suggested, consistent with previous studies (Grinshpun et al. 1997; Willeke et al. 1998), that only minor penetration and re-aerosolization losses may have occurred in the impingers.

All slit-to-agar (reference) colony counts were nonzero, so when calculating relative efficiency a zero value in the numerator indicated an observed relative efficiency less than 1 over the slit-to-agar count. However, these are censored values. Six of the 18 impinger relative efficiency values had zero colonies (3 from each impinger type), so we compared impinger efficiencies first by using the nonparametric Mann–Whitney *U* test. The test showed no significant difference in the impinger relative efficiencies. We then assigned the values of ½ the LOD for each censored data point, i.e., ½ of 1/(slit-to-agar count) for the trial and compared the impinger mean efficiencies using the two-sample *t* test after assessing normality. The *t* test also failed to show a significant difference in the impinger relative efficiencies ($p > 0.05$), and the data were pooled for the two impingers. The pooled collection efficiency was then 4.1% (95% CI 2.27–5.99%) relative to the slit-to-agar sampler.

For the trials in which two MCE filters sampled side-by-side, with each's location randomized across trials, the lack of a position-related difference was verified by conducting a paired-sample *t* test. The pair differences for the ten filter trials were approximately normally distributed as shown by the Shapiro–Wilk test ($p = 0.45$). The paired-sample t test failed to show a significant positional difference in the paired filter measures ($p > 0.05$), indicating good mixing in the chamber, and the filter measures were pooled. In contrast to the extremely low impinger relative efficiencies, pooled sampling efficiency values for MCE filters relative to the slit-to-agar sampler ranged from 89.0 to 189.6%. Mean relative sampling efficiency, compared to the slit-to-agar impactor, was 136.6% (95% CI 124.7–148.5%). Sample means and standard errors of the mean (SEM) are shown in Fig. 3.

Fig. 3

Air sampler efficiencies (mean ± SEM) relative to the slit-to-agar impactor

Full size image

In the 20 trials to assess the potential for re-aerosolization of spores from impinger suspensions, with 10 trials per impinger type, the culturable spore concentrations were seen to decrease by a mean of 89.96% for the AGI-30 and 89.51% for the BioSampler. While some CFUs were seen on the in-line filters placed downstream of the impingers, these accounted for less than 0.35% of the spores in the original suspension.

4 Discussion

These results demonstrate substantial and surprising differences in the sampling efficiencies of liquid impinger, slit-to-agar impactor, and MCE filter sampling methods when sampling airborne *C. difficile* spores under these conditions. The impingers were particularly ineffective. This suggested that *C. difficile* spores drawn into the impingers were either not captured in the liquid, were captured but became re-aerosolized by fluid turbulence, or were captured but rendered non-culturable by the collection sampling conditions. However, in-line filters on impinger samples demonstrated only minor penetration through these devices. *C. difficile* spores are approximately 1.4–1.9 μm in orientation-averaged aerodynamic size, and particles of this size are collected with high efficiency in all four collectors used. Our experiments demonstrated that spores were indeed captured in the impingers and were not re-aerosolized in significant numbers; rather, spores were rendered non-culturable by the impinger operation.

Sampling stresses that might affect culturability include osmotic shock, oxygen toxicity, desiccation, and mechanical stresses. All of these are known to affect vegetative organisms during air sampling (Kesevan et al. 2010) but are not generally associated with effects on bacterial endospores. Indeed, spores of most types, including *C. difficile*, have been shown to survive in the environment for long periods while subjected to desiccation, light exposure, atmospheric oxygen, and temperature extremes (Kim et al. 1981; Edwards et al. 2016). The extremely poor recovery of *C. difficile* spores from impinger fluid in these trials is in sharp contrast to the recovery of *Bacillus atrophaeus* spores, used as a surrogate for anthrax spores, when sampled using liquid impingers (Kesevan et al. 2010). The influences of each of these stressors on *C. difficile* culturability during air sampling by impaction, impingement, or filtration are an area deserving of additional research.

5 Study limitations

The scope of this study was necessarily limited to a selection of the air sampling methods commonly used for the bioaerosol assessment. We examined the *C. difficile* spore bioaerosol sampling efficiency of the two most commonly used liquid impinger bioaerosol samplers, one type of impactor and one type of filter medium (MCE). We used one impinger fluid (sterile PBS), one sampling duration, and one set of culturing conditions. We also used a non-toxigenic strain of *C. difficile* as a surrogate for toxigenic *C. difficile* strains and prepared and aerosolized the spores in essentially pure suspensions. Whether naturally occurring toxigenic organisms aerosolized from other media (e.g., when flushing a contaminated toilet) and sampled and cultured by other methods would have similar susceptibility to sampling stresses is unknown. The particular mechanisms affecting the culturability of captured *C. difficile* spores, and whether other endospore types might be affected, are also unknown. These and associated questions will require further study.

6 Conclusions

This study was the first to compare air sampling devices and methodologies in the sampling of a laboratory-generated aerosol of *C. difficile* spores. Surprisingly, impinger bioaerosol sampling as conducted in this work was not effective for characterizing airborne *C. difficile* spore concentrations. The two impingers had equivalent performance, which was extremely poor compared to both the slit-to-agar impactor and MCE filters. Therefore, impinger bioaerosol sampling could routinely fail to detect the presence of aerosolized *C. difficile* as demonstrated by the absence of *C. difficile* in 6 of 18 impinger trials despite the presence of numerous colonies on the slit-to-agar plate. Of the three methods, filter-based sampling using MCE filters with direct culture yielded the highest estimates of airborne spore concentrations and indicated that air sampling of *C. difficile* by slit-to-agar impaction, the most practiced method, may actually underestimate airborne concentration and thus fail to fully assess the airborne transport risk in healthcare settings. Filtration-type air samplers are far more widely available to health and safety professionals and industrial hygienists. Air sampling via filtration is also less costly and much simpler than slit-to-agar sampling in terms of equipment

requirements, consumables use, and sample storage. The mechanism by which *C. difficile* spores were damaged by impinger operation, and the rate at which damage occurs for captured spores are presently unknown. However, similar damage has not been observed in *B. atrophaeus* endospores. Additional research in each of these areas is needed. This study also demonstrated the effectiveness of *C. difficile*-selective chromogenic agar in the analysis of air samples using multiple air sampling media.

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