Effective Heat Sterilization in CO₂ Incubators

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In 1878, Sternberg showed that pathogenic bacteria (vegetative or non-spore forming cells) were killed in 10 minutes at a relatively benign temperature of 62°C to 70°C (143.6°F to 158°F), whereas spore formers required 5 minutes exposure to moist heat at 100°C (212°F). As early as the 1880s, comparisons of dry heat and moist heat processes were conducted on anthrax spores (Perkins, J. J., 1969). These early observations of the extreme physical and chemical resistance of bacterial spores, as compared to nonspore formers (vegetative cells), had important consequences. The use of spores from selected bacterial strains became the indicator organisms of choice for dry heat, steam, and other disinfection and sterilization processes.

Dry heat, recognized as an efficient process, evolved from simple ovens to double wall convection ovens to modern high-efficiency, forced air ovens. Dry heat was very effective for moisture sensitive items such as cutting edge instruments (scalpels), dry chemicals, oils, and glycerine. Early work with dry heat convection ovens showed that instruments and glassware were readily sterilized due to their excellent heat conducting properties. For packaged items, the slow and uneven penetration of dry heat and resultant long exposure times made the process less desirable than steam for hospital applications.

Moist heat processes, especially with the advent of the autoclave, became the sterilization method of choice in hospitals. This was because pressurized steam readily penetrated wrapped instruments and other packaged items, thus permitting rapid turnaround and an assurance of sterility. Settings of 15 to 20 lbs. (6.8 kg to 9.1 kg) pressure for 20 to 30 minutes became recognized as effective process parameters by the turn of the century. Unfortunately, steam must be contained in a pressure vessel, making it inappropriate for equipment surface sterilization. Dry heat specifications continued to be debated due to the poor penetration of dry heat for various load configurations. About 1930, an upper limit of 160°C (320°F) was set for dry heat cycles because instruments of the day could lose their temper at higher temperatures.
temperatures (AAMI ST-50, 1995). Empirical work showed that various bacterial spores could be readily killed at temperature ranges of 120°C to 180°C (248°F to 356°F), showing dry heat to be effective when exposure conditions were controlled. However, there were few heat transfer studies on loads, and a widely accepted standard of 1 hour at 160°C was adopted for instruments and instrument packs. The U.S. Pharmacopeia (USP) later recommended 170°C (338°F) for 120 minutes; the American Dental Association (ADA) currently recommends 160°C (320°F) for 120 minutes (AAMI ST-50, 1995). These recent guidelines, plus the early work done at the 120°C to 180°C (248°F to 356°F) range, show that no single time/temperature parameter was mandated. Quite simply, higher process temperatures meant faster instrument throughput, which was a pragmatic and often necessary approach in hospitals.

Historically, dry heat is a proven process. Long process times for wrapped loads currently limit the use of dry heat in hospitals. However, the dry heat process proves ideal for surfaces.

Incubators, such as the Thermo Scientific Steri-Cycle Series (figure 1), have been designed for dry heat sterilization through the following:

- Improved heat transfer systems that use mechanical air circulation
- Radiant wall heaters to replace electric coils
- High efficiency insulation and gasketing to minimize temperature loss and ensure uniformity

Considerations When Conducting Dry Heat Validations

The Association for the Advancement of Medical Instrumentation (AAMI) and ADA process guideline recommendations are directed to sterilization of wrapped loads for health care and industrial applications. While the principle of using dry heat to sterilize empty chambers is sound, the time/temperature guidelines are not applicable. Because sterilizing an empty incubator with shelves is strictly a surface process, validation testing to allow the use of fixed exposure cycles (time and temperature) is a forthright process. Bacterial spores calibrated for dry heat processes can be utilized directly on incubator chamber materials and the time required to achieve sterilization determined empirically.

Variables associated with the dry heat sterilization process are as follows (AAMI ST-50, 1995):

- **Temperature.** This is the most important variable; the effect of temperature on lethality is related to time.
- **Time.** This term refers to the “interval” or “exposure time” at a pre-determined temperature that will result in sterilization.
- **Airflow and Distribution.** Uniform distribution of air results in uniform transfer of heat throughout the incubator. Further, hot air flowing over surfaces reduces microorganism resistance by way of dehydration, which results in reduced sterilization times.
- **Load Configuration.** Load size and density are very important when considering dry heat sterilization of objects. An empty incubator presents a “no load” condition because the interior walls, shelf, and door components are readily heated by the circulating hot air.

Also critical is the indicator organism used to validate the system. Suspensions of Bacillus subtilis spores calibrated for dry heat processes are used to validate sterilization of the chamber interior (U.S. Pharmacopeia XXIV, Chapter 1035). Note that spores of Bacillus stearothermophilus are only specified as indicators for use under saturated steam conditions and are not the indicator of choice for dry heat processes (ANSI/AAMI/ISO 11134, 1995). Typically, commercially available dry heat biological indicators, which consist of Bacillus subtilis spores inoculated onto filter paper strips and packaged in glassine envelopes, are used to monitor the sterilization of dry heat loads in health care and industrial settings (U.S. Pharmacopeia XXIV, Official Monograph). These commercially available...
Materials. Stainless steel, glass, and silicone gasket components were cut into pieces to act as carriers onto which the resistant spores were to be inoculated. The carriers were made from the following materials:

- Grade 304 stainless steel
- High quality glass from microscope slides
- Silicone gasket material used as the door interface

Carriers were washed to simulate normal incubator cleaning prior to inoculation.

Resistant Spore Preparations. Suspensions of Bacillus subtilis var. niger spores – ATCC #9372 (American Type Culture Collection, 1999) or NRRL B4418 – were purchased from three prominent manufacturers in the United States. The most resistant suspension available was requested from each vendor at the time of purchase. Spore preparations met resistance guidelines recommended in the USP Chapter 1035; D160C values were as follows:

- 1.4 minutes for Vendor #1
- 2.6 minutes for Vendor #2
- 2.0 minutes for Vendor #3

D160C refers to decimal reduction and describes the time, in minutes and at 160°C (320°F), required to reduce a population of organisms by 90% (or one log). A six-log reduction cycle reduces the population of resistant spores by 99.9999%.

Test Methods

Temperature Mapping – Minimum Chamber Temperature and Uniformity. Because temperature is critical in dry heat processes, the chamber was thoroughly mapped with thermocouples to measure uniformity at target temperatures and to identify the coolest, and therefore least lethal, location in the incubator. The lowest temperature was the benchmark temperature used when conducting the sterilization studies (figure 2).

Lethality tests, using inoculated materials, were conducted in an area of constant temperature (middle shelf) that was adjusted to mimic the minimum temperature established for the incubator. The temperature used for testing was 133.5°C (275°F). A typical Thermo Scientific Steri-Cycle CO₂ Incubator exhibits a minimum chamber temperature of 133.5°C (275.4°F) during the sterilization cycle. By using a Steri-Cycle unit for these tests, the inoculated carriers were subjected to any normal temperature vagaries characteristic of each test run.
Inoculation of Carriers. Carriers made from each of the three materials—stainless steel, glass, and silicone—were inoculated with each of three commercial suspensions of B. subtilis spores and allowed to dry. Carriers were stored under ambient conditions and used within 16 hours of preparation.

Control carriers were prepared at the same time. These carriers remained under ambient conditions until the end of each study. Upon completion of each study, the carriers were processed to remove and count the number of viable spores. Counts performed at the end of each test period are more indicative of the number of viable cells undergoing the validation process, thus ensuring a more accurate lethality calculation.

Exposure – Rationale. Tests for establishing a sterilization cycle (minimum six-log reduction of indicator spores) will be conducted using growth/no growth criteria as evidence of complete kill of inoculated carriers.

Sets of inoculated carriers (>1E6 spores per carrier) will be subjected to increasing process times at the incubator target temperature. As exposure time increases, a point will be reached where no viable spores survive the process. This will be illustrated when exposed, inoculated carriers fail to show growth after being placed in nutrient recovery medium and incubated for seven days.

Using the results from several growth/no growth studies, a cycle time that consistently achieves sterilization can be established. Such cycle determinations are possible because B. subtilis spores exposed to dry heat processes die in a linear manner, which is affirmed by data supplied with vendor spores.

Exposure – Method. Direct inoculation of incubator walls and shelves, followed by spore recovery using swabbing methods, can be used to characterize the lethal processes during a cycle. However, this method shows an inherent variability and less than 100% recovery.

The method of choice in this study was the following:

- Inoculate pieces of the construction materials with >1E6 resistant indicator spores.
- Subject the inoculated materials to dry heat cycles.
- Culture each inoculated piece in liquid growth media.

The following procedure was used.

1. The incubator was turned on and allowed to reach the setpoint and stabilize as determined by use of calibrated thermocouples.

2. All inoculated carriers for each study were quickly placed into the stabilized incubator chamber. Each set of carriers consisted of

- 9 glass carriers – 3 inoculated with Vendor #1 spores, 3 inoculated with Vendor #2 spores, and 3 inoculated with Vendor #3 spores
- 9 stainless steel carriers inoculated as above
- 9 silicone carriers inoculated as above

3. After the inoculated carriers were exposed for a minimal time period, the door was quickly opened and one complete set of carriers removed for cultivation.

4. Carriers were immediately cultured as noted in the Culturing and Incubation section.

5. Another carrier set was removed and cultured after an additional exposure segment (i.e., 7 minutes).

This procedure continued until all sets of carriers were removed and cultured. Testing was conducted simultaneously on sets of inoculated carriers so that spore or materials differences could be observed. Also, by testing all components and spores simultaneously, any effect from door openings or subtle incubator performance variations would be negligible.

Culturing and Incubation. Each exposed carrier was cultured into soybean casein digest broth immediately upon removal from the incubator. As recommended in USP, carriers were incubated for 7 days at 30°C to 35°C (86°F to 95°F) (U.S. Pharmacopeia XXIV, Chapter 55). This incubation period is necessary to ensure that sublethally injured microorganisms have adequate time in a nutritive environment for repair and outgrowth. The 48 hours generally allowed for outgrowth of routinely cultured organisms (non-injured organisms) is not adequate for the development of lethal cycles.

1 “Greater than or equal to” 1E6 or 1 x 10^6 or 1,000,000 spores
2 Injured organisms are most likely to survive and grow if placed directly into liquid growth medium (soybean casein digest broth is recommended). An accurate endpoint of the lethality cycle can be determined using this method.
Using this process, Steri-Cycle CO2 Incubators will eliminate vegetative microorganisms (e.g., E. coli, Pseudomonas species, staph, strep, and Mycoplasma species) as well as fungal spores (e.g., Aspergillus and Penicillium species) before a half cycle is reached (Sykes, G., 1965).

A temperature profile of the complete sterilization process shows an additional margin of effectiveness (figure 4). During the gradual ramp up to the target temperature and the early segments of the cool down cycle, the considerable heat near the target temperature also contributes to microbial destruction.

Conclusion

With the validation of a sterilization cycle specific to Thermo Scientific Steri-Cycle CO2 Incubators, the user can be assured of complete contamination elimination. The cycle was established using nationally recognized guidelines and commercial spore suspensions calibrated specifically for dry heat validations. Six-log reduction times, which result in the destruction of high levels of resistant indicator spores, were more than doubled to meet stringent sterilization standards established for the health care industry. As a result, the user can initiate the single pre-programmed sterilization cycle and return to a sterile incubator after a few hours. No pans of water are required, and the maintenance-free stainless steel and glass chamber

Test Results

Figure 3 summarizes the results of triplicate test runs, each comparing all materials versus all vendor spore preparations. At the extreme, spores from Vendor #1 demonstrated a uniformly lower resistance to the dry heat process, while spores from Vendor #2 showed the greatest resistance. These results paralleled the resistance data supplied by the respective vendors.

The average values refer to the combined average of all vendor spores for each material type. With respect to materials differences, spores inoculated onto silicone material required a slightly longer exposure time to ensure complete kill of spores. Overall, the three materials are quite similar with respect to the effect they exert on surface microorganisms being subjected to a dry heat process. These data were used to establish a sterilization cycle. The longest average exposure time to achieve a six-log reduction on the worst case material was 41 minutes (Vendor #2, spores on glass). To ensure the greatest margin of sterility assurance, this 41 minute cycle was doubled to yield an 82 minute cycle (figure 4).

This conservative approach, essentially a 12-log reduction, is advocated by AAMI for surgical instruments and implantable devices. To create an additional sterilization margin, the net cycle time at sterilization temperature was extended to 120 minutes.

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ensures that no corrosive copper salts or toxic chemicals need to be removed.

The efficient directed air distribution system that provides precise temperature uniformity for cell culture applications also ensures uniform distribution of sterilizing dry heat.

We have selected one of the oldest recognized methods for sterilization and has effectively adapted the process to the complete line of Thermo Scientific Steri-Cycle CO2 Incubators. The result is an efficient, reliable, and convenient process for tomorrow’s laboratory.

### About the Author

Dr. Dalmaso earned a PhD in Microbiology and Immunology from The Ohio State University. He has more than 30 years of experience in university teaching and research, medical microbiology, food technology, and sterilization cycle development in the medical device and pharmaceutical industries. Apex Laboratories, Inc. is an independent laboratory that provides contract and validation services to the food, pharmaceutical, and medical device industries.

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**figure 4**—Typical control temperature profile of the sterilization process
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