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▲⟨56⟩ METHODS FOR DETERMINATION OF RESISTANCE OF MICROORGANISMS TO STERILIZATION PROCESSES

INTRODUCTION

Sterilization relies on the reliable destruction of microorganisms present in/on the materials being sterilized. Information regarding the resistance of the bioburden to the sterilization process (with the possible exception of overkill processes) is essential to confirm the efficacy of the sterilization process (see *Sterilization of Compendial Articles* ⟨1229⟩) and is also a required component of a parametric release program (*1*,*2*). Spores exhibit inherent and varying levels of resistance to all modalities of sterilization. There are limited resistance data available for some spore-forming and pathogenic microorganisms; however, for the vast majority of microorganisms (including most spores), there is no data available regarding their susceptibility to sterilization processes when present in or on pharmaceutical articles (*3*). Furthermore, wild type microorganisms and spores derived from manufacturing processes are likely distinct from their reference-standard culture-collection counterparts and may exhibit differing levels of resistance to sterilization processes (*3*). This chapter outlines methods that can be used to determine the presence of spores and the resistance of microorganisms, spores, and biologic indicators (BIs) to moist heat sterilization, including variations of the boil test mentioned in *Moist Heat Sterilization of Aqueous Liquids* ⟨1229.2⟩ and *Monitoring of Bioburden* ⟨1229.3⟩. The methods included in this chapter are not intended for use in radiation sterilization where the procedures described in ISO 11137 are to be followed (*4*).

GENERAL PROCEDURES

Carry out the tests under conditions designed to avoid extrinsic microbial contamination of the materials to be examined. The precautions taken to avoid contamination must be such that they do not affect the microorganisms that are recovered in the test. If the product to be examined has antimicrobial activity, insofar as possible, this should be removed or neutralized during the presence or absence test for spores. If inactivators are used for this purpose, their efficacy and their nontoxicity to the microorganisms must be demonstrated. If surface-active substances are used for sample preparation, their nontoxicity to the microorganisms and compatibility with any inactivators used must be demonstrated.

Preparation of the Sample

The method for sample preparation depends on the physical characteristics of the pre-sterilization material to be tested. For aqueous products, 10-mL samples should be taken from closed primary containers. Composite sampling may be used. For solid, water-insoluble, or immiscible products, follow the sample preparation details provided in *Microbial Enumeration Tests* ⟨61⟩. Samples should be aseptically transferred (either from 1 unit or composite) to sterile, screw-capped tubes. A minimum of 10 samples should be tested to minimize variability.

PRESENCE/ABSENCE TEST FOR SPORES (ALL STERILIZATION PROCESSES EXCEPT FILTRATION AND RADIATION)

A universal assumption in overkill sterilization processes is that the resistance of the bioburden microorganisms as compared to the BI is negligible because the BI is specifically chosen as a "worst case" challenge to the sterilization process (*1*). This is predicated upon an assumption of the absence of spores in the pre-sterilization bioburden. A test in which bioburden samples are exposed to 70°–75° for 30 min can be used to confirm the absence of spores. Vegetative microorganisms are seldom resistant to temperatures >70° and are killed under these conditions. However, under these temperature conditions with the presence of non-nutrient germinants (agents not necessary for growth but which promote germination of spores, such as Ca-DPA and dodecylamine), spore germination is promoted by heat activation. This test is suitable where the presence of spores in the pre-sterilization bioburden is associated with a greater chance for survival (*5*). When spores are detected, resistance testing as outlined below for moist heat sterilization or in ISO 11138 series of standards (*6*,*7*) should be conducted to confirm that process lethality is sufficient.

Heat Treatment

Transfer the screw-capped tubes to a water bath, heat the contents to 70°–75°, and hold at this temperature for 30 min.

Recovery and Enumeration of Spores

Immediately cool the product to 30°–35°, and aseptically add sterile calcium dipicolinic acid (Ca-DPA) and dodecylamine (non-nutrient germinants) in Buffered Sodium Chloride–Peptone Solution pH 7.0 or in Phosphate Buffer Solution pH 7.2 to generate concentrations of 60 and 1.2 mM, respectively. Maintain the solutions at 30°–35° for 30 min. Use either membrane filtration or plate-count methods per ⟨61⟩ for

the recovery and growth of spores with *Soybean–Casein Digest Agar* containing 60 mM Ca-DPA and 1.2 mM dodecylamine, incubated at 30°–35° for 5–7 days.

Positive and Negative Controls

To verify efficacy of heat treatment, a positive control is included by inoculating at least one additional test sample with a countable number of spores such as *Bacillus subtilis*. There must be growth of microorganisms. To verify testing conditions, a negative control is included using the chosen diluent in a screw-capped tube in place of the test sample. There must be no growth of microorganisms.

Growth Promotion of Media

Test each batch of *Soybean–Casein Digest Agar* containing 60 mM Ca-DPA and 1.2 mM dodecylamine per *Microbial Enumeration Tests ⟨61⟩*, *Table 1*.

Suitability of Recovery and Enumeration

Perform method suitability per ⟨61⟩ by adding a sufficient volume of microbial suspension of *Bacillus subtilis* ATCC 6633, NCIMB 8054, CIP 52.62, or NBRC 3134 to the cooled product and demonstrating acceptable recovery on *Soybean–Casein Digest Agar* containing 60 mM Ca-DPA and 1.2 mM dodecylamine.

Interpretation of Results

The presence of colony forming units suggests that spores are on/in the product and more definitive estimation of their resistance should be considered (see below).

RESISTANCE ESTIMATION (MOIST HEAT PROCESSES ONLY)

This test method, also known as Boil Test, can be applied to estimate moist heat resistance of spores present in pre-sterilization product. Initially, a range of times can be used to estimate moist heat resistance (*Preliminary Resistance Estimation*). The use of a single time period tailored to the pre-established limits for resistance (*Maximum Resistance Estimation*) can be used to support parametric release to confirm sterilization process suitability. Either of these tests can be performed with parallel testing of a biological indicator microorganism for comparison purposes.

Preliminary Resistance Estimation

Transfer samples in screw-capped tubes to a water bath, heat the contents to 95°–100°, and incubate at this temperature using a range of time periods (see *Table 1*). *Recovery and Enumeration of Spores*, *Positive and Negative Controls*, *Growth Promotion of Media*, and *Suitability of Recovery and Enumeration* are performed as described above.

INTERPRETATION OF RESULTS

The estimated moist heat resistance of spores is established by the maximum time where colony growth is determined as shown in *Table 1*. Adjustments in dwell time (shorter or longer) at 95°–100° can be used to accommodate the microorganisms detected.

Maximum Resistance Estimation

Transfer samples in screw-capped tubes to a water bath, heat the contents to 95°–100°, and incubate at this temperature for the desired time. *Recovery and Enumeration of Spores*, *Positive and Negative Controls*, *Growth Promotion of Media*, and *Suitability of Recovery and Enumeration* are performed as described above.

INTERPRETATION OF RESULTS

The presence of colony forming units suggests that spores are present with a level of moist heat resistance to sterilization and requires further investigation.

Table 1. Moist Heat Resistance Estimation from Boil Test

Exposure Time at 95°–100° with Positive Growth (min)	Approximate $D_{121°}$-Value (min)
10	≥0.1
20	≥0.2
30	≥0.3
40	≥0.4
50	≥0.5

D-VALUE DETERMINATION (MOIST HEAT PROCESSES ONLY)

The determination of *D*-value for microorganisms and BIs is a required aspect of sterilization cycle development and validation (*1,8*). The established procedures for this determination are focused on moist heat processes performed near 121° and rely upon equipment specifically designed to operate with saturated steam. Accurate resistance determination for biological indicators and bioburden isolates from moist heat processes operating at lower temperatures can benefit from the alternative practices described below.

D-value Determination for >110° Processes

The methods described in *Biological Indicators—Resistance Performance Tests ⟨55⟩* should be followed. Additional details regarding the procedures and equipment can be found in ISO 18472, *Sterilization of health care products—Biological and chemical indicators—Test equipment* (*9*).

D-value Determination for ≤110° Processes

The apparatuses for *D*-value determination at 121° utilize saturated steam and resemble steam sterilizers in their design and operation. With the use of sterilization processes at temperatures ≤110°, adaptation of the test equipment and methods to better suit the use of lower temperatures is appropriate. Adjustments in dwell time (shorter or longer) and operating temperature may be necessary to accommodate the microorganism being tested (*10*). These methods apply directly to BI's used for low temperature moist heat processes and must be adapted for use with bioburden isolates.

PROCEDURE

Carry out the tests for *D*-value at sterilization conditions consistent with those intended for use. Use 20 replicate (BI or bioburden isolate) test sample items subjected to NLT 5 exposure conditions for a total of 100 tests. The number of exposure conditions is chosen to provide a range of observations from NLT 1 *D*-value below the expected survival time through NLT 1 *D*-value above the expected kill time. Place each group in a separate suitable sample holder that permits each sample to be exposed to the prescribed temperature condition in a water bath at 95°–100° (other temperatures can be utilized as necessary). Check the apparatus for operating parameters using sample holders with dummy test samples. Select a series of sterilizing times in increments starting with the shortest time for the samples to be tested. The differences in sterilizing times over the series are as constant as feasible, and the difference between adjacent times is NMT 75% of the expected *D*-value. Test procedures for the evaluation of microbial resistance are defined in the ISO 11138-series standards (*6,7*). The test methods and carriers used may be adapted to the specifics of the microorganisms being evaluated.

For microorganisms that are spores in suspension, the test is conducted using appropriate serial dilutions predicated upon the stated spore titer of the suspension in *Sterilized Purified Water* in a sterile tube.

RECOVERY

After completion of the sterilizing procedure and within a noted time (NMT 4 h), aseptically remove and add each sample to a suitable medium (see *Sterility Tests ⟨71⟩, Culture Media and Incubation Temperatures*), submerging the unit completely in a suitable tube. For self-contained BIs, the paper strip is immersed in the self-contained medium according to manufacturers' instructions, within a noted time of NMT 4 h. For insoluble items inoculated with a spore suspension, aseptically transfer these units individually to a suitable medium (see media in *⟨71⟩*) supplemented to contain 60 mM Ca-DPA and 1.2 mM dodecylamine and submerge the items completely in the medium. When a sealed aqueous filled container has been inoculated, test the units individually, as described in *⟨71⟩*.

Incubate each tube at the optimal recovery temperature. For bioburden isolates, recovery methods and media may be modified as needed. Observe each inoculated medium-containing tube at appropriate intervals for a total of 7 days after inoculation. Where growth is observed at any observation time, further incubation of the test sample(s) concerned may be omitted. Note the number of samples showing no evidence of growth at any time.

Where *Clostridium sporogenes* or another anaerobic microorganism is used as a BI, methods for preparation, inoculation, recovery, and media must be adapted to accommodate the use of these anaerobic spore-formers.

CALCULATION

The determination of *D*-values of microorganisms can be performed using the limited Spearman-Karber procedure, survival curve method, or Stumbo-Murphy-Cochran procedure (*3,11,12*). When a BI has been purchased, use the same method as that defined by the BI manufacturer to subsequently determine *D*-values. The use of an alternate method can result in differences that may be an artifact of the method rather than a variation in the performance of the BI.

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