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D-Value Determination Of Environmental Isolates From Sterile Manufacturing

W.Jamil[1], A. Zahid[2,] A.Jabbar[3], Z.Ali[4], S. Faraz Ikram[5]
[1,2,3,4,5] *Hamdard University Karachi Pakistan*

Abstract: *Aqueous spore suspensions of 3 different species of Bacillus isolated from Sterile manufacturing were heated at different temperatures for various time intervals in a water bath, counted by plate count method on Tryptic Soy Agar (TSA), and incubated at 32.5 °C. Remaining or resistant spore of isolated Bacillus species form sterile manufacturing of form colonies on TSA. D-Value, the decimal reduction time (time required at a given temperature for destruction of 90% of the cells), when survivors were colonized on TSA and the calculated D-Value are for SMF-B-001, 13.19 ± 0.05919, for SMF-B-002, 12.25 ± 0.051919 and SMF-B-003, 14.09 ± 0.05319 respectively. Recovery on TSA resulted in reduced decimal reduction time showing that damage occurred early in the thermal dealing. Heat sensitive spores showed sensitivity also to other post processing environmental factors such as incubation temperatures, a pH of 7.3 ± 0.3 for the medium.*

Keywords: *Spore= Most resistant form of vegetative cells ,TSA= Tryptic Soy Agar, Incubation= specicific temperature or condition which helps microorganism to grow, SMF= sterile manufacturing facility.*

I. Introduction

A D-value is the time in minutes, at a specific temperature to diminish or reduce the existing microbial population to 1-log or 90%. There are several different methods for D value determination. Three most common methods are listed below:

Method A, survivor curve method
Method B, the fraction negative method
Method C, over kill approach

1.1 Method A: Survivor Curve Construction

The survivor curve edifice method for microbiological performance qualification consists of exposing inoculated process challenge devices (PCDs) heated at specified temperature to different intervals of time, removing the microbial culture from PCD and enumerating the number of surviving organism in each tube. This data generates a survivor count that can then be used in developing a survivor curve.

1.2 Method B: Fraction Negative

The fraction negative method of microbiological performance qualification consists of exposing inoculated process challenge device heated at specified temperature to different intervals of time, removing the microbial culture from PCD and incubated directly to required temperature, checking the growth media for signs of growth and rating the sterility tests as growth/no growth of microbial culture. The proportion of tubes with no growth compared to those with growth and the corresponding time intervals are utilized to determine the D value.

At least five fractional studies should be conducted at graded exposure time (e.g. 0, 2, 4, 6, 8…. minutes) once the sub lethal cycle parameters are selected, all the fractional studies are performed using same cycle parameters. At least one study must results in growth of all test organisms, two studies must results in fractional growth of the test organism and two studies must results in the complete inactivation or no growth of all test organisms.

1.3 Method C: Overkill

This is the most common and cost effective method utilized in the industry. This method involves determination of the minimum time of exposure to sterilization process at which there are no survivors. The specified exposure time should be at least double this minimum time i.e. Sterility assurance level (SAL) + safety factor. A cycle of short duration from which survivor can be recovered should also be run to demonstrate the adequacy of the recovery technique.

1.4 Comparison Of Methods

The fraction-negative method is the preferred method of microbiological performance qualification due to both its simplicity as well as the complexity of the survivor curve method. When expense is an issue, the

fraction-negative method is again the method of choice, unless an in-house laboratory can be utilized. Where as if the sensitivity of material/product being sterilized is not a matter of concern, then overkill approach is a method of choice.

Before it the D-value never had been determine on sterile manufacturing facilities Isolates. All the work on D-Value determination has been performed and researched on food and Hospital sectors. This study may help the Pharma sector to know about the D-value determination of isolates who recover from the sterile manufacturing facilities.

II. Materials & Methods

Test tubes, water bath, Petri plates 90mm, Purified water, Tryptic soya broth, Tryptic soya agar, saline tubes, and pipettes, Cultures: selected Environmental isolates from sterile manufacturing area of.

2.1 Instrument

Autotoclave for sterilizing the media and diluents. Hot air sterilizer for the sterilizing the glass ware, Micropipettes for the dilution and pouring, Laminar flow cabinet or bio safety cabinet for the testing, Microscope, incubators for incubation purpose.

2.2 Reagents

Tryptic soya agar, Tryptic soya Broth, Sodium chloride, Distilled water, Iso propyl alcohol, crystal violet, safranine, iodine, ethanol/methanol, malachite green. Ethyl alcohol, ammonium citrate monohydrate, cider wood
oil.

2.3 Environmental Monitoring

Perform environmental monitoring of pre-selected rooms of Sterile Area Manufacturing and incubate the plates in incubator at 32.5 °C ± 2.5 for 72 hours. After 72 hours remove the plates from incubator and count the colonies of microorganisms and select three different organism one from each plate.
Select the colony by marking round with the help of permanent marker and sub culture it on fresh plate of TSA and incubate incubator at 32.5 °C ± 2.5 for 24 hours for the colonial characteristic of selected microorganism. After 24 hours, perform gram staining.

2.4 Procedure for Gram Staining

- To prepare the smear with the help of a dropper add a drop of saline to a clean slide. Take a sample of a single bacterial colony and mix it into the saline. Let air dry until a white film appears. Heat fix by passing the slide through a flame a few times.
- Cover the smear with crystal violet for 60 seconds.
- Wash the slides with low stream of distilled water for 15 seconds.
- Now pour Gram Iodine on smear for 60 seconds.
- Wash the slides with low stream of distilled water for 15 seconds.
- Use 95 % ethanol to decolorize the smear by dropping on smear until the ethanol runs clear about 20 to 30 seconds.
- Wash the slides with low stream of distilled water for 15 seconds.
- Now use safranin as a counter stain for 30 seconds.
- Wash the slides with low stream of distilled water for 15 seconds.
- Allow the slide to air dry.
- Arrange Microscopy on oil immersion lenses of100 xs to differentiate the microbes into gram negative or gram positive
- Gram positive are Purple in color and gram negative are pink in color.

2.5 Culture Preparation

- After Determine their colonial and microscopic morphology of 3 different isolates from Sterile Area Manufacturing
- Perform heat shock method to isolate most resistant environmental isolate as follows:
- Prepare 10^6 cfu / ml suspension of isolate. Heat the tube containing suspension in a water bath at 95 to 100°C for 15 minutes (heat shock), starting the time when the temperature reaches 95°C. Cool rapidly in an ice bath at 0-4°C. Perform serial dilution of suspension and enumerate the spore count.
- Assign reference number to selected culture as follows. Assign first three letters representing facility from which culture isolated followed by single letter representing genus followed by serial number.

- For Example: SMF-B-001

SMF = Sterile manufacturing facility
B = Bacillus
001 = Serial number
Prepare 150 tubes of 10^6 cfu/mL for each culture in TSB

2.6 Procedure

- Prepare a process challenge device by setting a water bath at boiling temperature i.e. 100 °C.
- Make 15 set of 10 test tube of each culture in TSB broth.
- Mark each set of tubes (10 tubes) with the time of exposure.
- As soon as the temperature of water bath reaches to 100 °C place all set of tubes in water bath.
- Remove first set of tubes at 0 minutes.
- After every minute, remove one set of tube until 15 minutes.
- Incubate each set for growth at 32.5 ± 2.5 °C for 7 days.
- Determine the growth and no growth for each set and then follow the calculation procedure.

III. Indentations And Equations

D-Value Determination Formula

$$T = Tk - (\frac{\delta}{10} \times \sum_{1-1}^{k-1} fl),$$

T is Time
T_k =when no growth appears in all specimen and
δ = represent constant intervals between successive exposures.

$$D = \left[\frac{T}{\log N\,o + 0.2507}\right]$$

T= time of exposure and log N o is the initial Spore count of organism.

$$Vt = \frac{\delta^2}{n^2(n-1)} \times \sum_{1=1}^{k-1} fl\,(10 - fl).$$

Time of making this test calculate the variance of T, V_t by the equation. In which δ represent constant interval between successive exposures, as define above

$$ST = \sqrt{Vt}$$

The standard deviation St is the square root of variance:

Calculate the lower and upper 95% confidence interval (approximate CL) for the D-Value by the equation Approximate CL for D = (T ± 2sT/log N0 + 0.2507).

IV. Result & Discussions

The Response of all 10 specimen's growth

Name of Isolate: Bacillus								
Code No: SMF-B-001, SMF-B-002, SMF-B-003			**Microscopic Morphology:** Gram Positive Bacilli					
Methods of D-value: Fraction negative method			**Assayed Resistance:** 100°					
SMF-B-001			**SMF-B-002**			**SMF-B-003**		
Initial spore (N_o): 636 x 10^5 cfu **Log of N_o:** 7.80			**Initial spore (N_o):** 512 x 10^5 cfu **Log of N_o:** 7.70			**Initial spore (N_o):** 525 x 10^5 cfu **Log of N_o:** 7.72		
F No*	**Exposure time (min)**	**Negative fraction / 10 specimen**	**F No***	**Exposure time (min)**	**Negative fraction / 10 specimen**	**F No***	**Exposure time (min)**	**Negative fraction / 10 specimen**
F1	1	0/10	**F1**	1	0/10	**F1**	1	0/10
F2	2	0/10	**F2**	2	0/10	**F2**	2	0/10
F3	3	0/10	**F3**	3	0/10	**F3**	3	0/10
F4	4	0/10	**F4**	4	0/10	**F4**	4	0/10
F5	5	0/10	**F5**	5	0/10	**F5**	5	0/10
F6	6	0/10	**F6**	6	0/10	**F6**	6	0/10
F7	7	2/10	**F7**	7	0/10	**F7**	7	2/10
F8	8	2/10	**F8**	8	0/10	**F8**	8	2/10
F9	9	3/10	**F9**	9	2/10	**F9**	9	3/10
F10	10	4/10	**F10**	10	6/10	**F10**	10	4/10
F11	11	6/10	**F11**	11	7/10	**F11**	11	6/10
F12	12	8/10	**F12**	12	8/10	**F12**	12	8/10
F13	13	10/10	**F13**	13	8/10	**F13**	13	10/10
F14	14	10/10	**F14**	14	10/10	**F14**	14	10/10
F15	15	10/10	**F15**	15	10/10	**F15**	15	10/10
F16	16	10/10	**F16**	16	10/10	**F16**	16	10/10

F17	17	10/10	**F17**	17	10/10	**F17**	17	10/10
F18	18	10/10	**F18**	18	10/10	**F18**	18	10/10
F19	19	10/10	**F19**	19	10/10	**F19**	19	10/10
F20	20	10/10	**F20**	20	10/10	**F20**	20	10/10
***F No. = Fraction number**								
D_{100} of isolate: 1.63 **Lower Limit at 95% confidence:** 13.14 **Upper Limit at 95% confidence:** 13.23			**D_{100} of isolate:** 1.54 **Lower Limit at 95% confidence:** 12.208 **Upper Limit at 95% confidence:** 12.291			**D_{100} of isolate:** 2.25 **Lower Limit at 95% confidence:** 13.03 **Upper Limit at 95% confidence:** 13.14		

V. Conclusion

D- Value of environmental isolates from sterile manufacturing area isolates on frequently basis. That is why the isolates from sterile manufacturing area of determined and calculated using limited spearman Karber method. Three bacillus species were selected from 3 different room of from sterile manufacturing area of for the same.

The D-Value of bacillus spp (SMF-B-001), (SMF-B-002) and (SMF-B-003) were identified as 1.63, 1.54 and 2.25 at 100 C.

This determined value is much lesser than the D-Value of most resistant specific of bacillus i.e. 2.5 minutes at 121 C.

However our autoclave cycle were developed at over killed approach therefore there is no risk of autoclave cycle parameter set for production autoclave and Quality control autoclave that used to run cycle at D-value of most resistant species, which is Geobacillus stereothermophillus.

This D-Value calculation might be utilized for the setting of cycle parameters (production autoclave other than the standard one. i.e. 15 minutes at 121 C especially for products that are thermo labile.

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References

[1] P. Thereza Christina Vessoni, Irene A. Machoshvili, Maria Elena Santos Taqueda, and Carlos Augusto Montenegro Ferraz. Bacillus Stearothermophilus Sporulation Response to Different Composition of Media to evaluate the effectiveness of 11 commonly used ingredients to improve Bacillus stearothermophilus ATCC 7953 sporulation. PDA J Pharm Sci Technol 52 1998.198-208.

[2] I I.R, A.E Oji, O.K Nwosu, E.S Amadi. Antimicrobial activiy salvon, Izal and Z-germicide Against clinical isolates of peseudomona aeruginosa from hospitals wards.European Jouranl of dentistry and medicine 3 (1) 2011, 32-35.

[3] O.OA, AB Olayemi, KIT Eniola, OA Adeyeba. Anti bacterial activity of some selected disinfectant regualrly used in hospitals.African Journal of Clinical and Experimental Microbiology 5(1) 2004, 126-130.

[4] B.Maria Lucia Morcerf, Vania Lucia Silva1, Felipe Augusto Sa Moreira1, Girlene Alves Silva2 and Claudio Galuppo Diniz. Antimicrobial resistance and disinfectants susceptibility of persistent bacteria in a tertiary care hospital.Journal of Microbiology and Antimicrobials Vol. 2(8), 2010, 105-112.

[5] G.Masood, Zahra Azimi. Isolation Identification and Antimicrobial Susceptibility of Pseudomonas spp. Isolated from Hospital Environment in Tonekabon, North of Iran Journal of Applied & Environmental Microbiology,2 (4), 2014, 97-101.

[6] A.Ranya Aly, Safinaz Aly Mady, Hoda H. Yusef, and Soraya Sabry. Determination of decimal reduction time (D-value) of chemical agents used in hospitals for killing airborne isolated bacteria Due to the growing numbers of infection outbreaks in hospitals. African Journal of Microbiology Research Vol. 7(26), 2013 , 3321-3330.

[7] D.R. Fenlon ,J. Wilson and W. Donachie. The incidence and level of Listeria monocytogenes contamination of food sources at primary production and initial processing .Listeria monocytogenes was isolated in low numbers from a variety of environmental samples associated with the primary production of food, including vegetation, faeces and meat. Journal of Applied Bacteriology Volume 81(6), 1996, 641–650.

[8] C.Harriette, Krönig and Paul. The Process of Disinfection by Chemical Agencies and Hot Water were the first to study the process of disinfection by making quantitative observations at intervals during its progress. Journal of Hygiene ,10(02)1910, 237-286.

[9] Ana Cláudia N.F. Spinelli, Anderson S. Sant'Ana, Cristiana P. Pacheco-Sanchez, Pilar R. Massaguer. Influence of the hot-fill water-spray-cooling process after continuous pasteurization on the number of decimal reductions and on Alicyclobacillus acidoterrestris CRA 7152 growth in orange juice stored at 35 °C.International Journal of Food Microbiology, 137, (2–3),28, 2010, 295-298.

[10] G.Alonzo A. Influences of heating temperature, pH, and soluble solids on the decimal reduction times of acid-adapted and non-adapted Escherichia coli O157:H7 (HCIPH 96055) in a defined liquid heating medium. International Journal of Food Microbiology, 160, (1), 2012,50-57.

[11] M.Celenk, Ayse Handan Baysal. Effect of sporulation medium on wet-heat resistance and structure of Alicyclobacillus acidoterrestris DSM 3922-type strain spores and modeling of the inactivation kinetics in apple juicelicyclobacillus acidoterrestris is a spoilage bacterium in fruit juices leading to high economic losses. International Journal of Food Microbiology, (189) 2014,82-88.

[12] R.Clémence, Jean-Baptiste Denis, Isabelle Albert, Frédéric Carlin. A meta-analysis accounting for sources of variability to estimate heat resistance reference parameters of bacteria using hierarchical Bayesian modeling: Estimation of D at 121.1 °C and pH 7, zT and zpH of Geobacillus stearothermophilus redicting microbial survival requires reference parameters for each micro-organism of concern. International Journal of Food Microbiology, 161, (2), 1, 2013,112-120.

[13] M.Celenk, Ayse Handan Baysal. Effect of sporulation medium on wet-heat resistance and structure of Alicyclobacillus acidoterrestris DSM 3922-type strain spores and modeling of the inactivation kinetics in apple juiceAlicyclobacillus acidoterrestris is a spoilage bacterium in fruit juices leading to high economic losses. International Journal of Food Microbiology, Volume 189, 2014 ,82-88.

[14] N.Truls, Karl Eckner, Ole-Johan Røtterud. The effect of blast chilling on occurrence of human pathogenic Yersinia enterocolitica compared to Campylobacter spp. and numbers of hygienic indicators on pig carcasses. International Journal of Food Microbiology, 123,(1–2, 3), 2008,130-133.

[15] N.Truls, Karl Eckner, Hilde Kristin Høidal, Ole-Johan Røtterud. occurrence of Yersinia enterocolitica and Campylobacter spp. in slaughter pigs and consequences for meat inspection, slaughtering, and dressing procedures. International Journal of Food Microbiology.80 (3),2003, 231-240.