DISINFECTNAT EFFICACY STUDY BY TUBE DILUTION METHOD

1.0 PRE-APPROVAL

Signing of this approval page of protocol indicates agreement with the validation approach described in this document.

Name	Department/Designation	Signature	Date
Prepared By:			
Reviewed By:			
Approved By:			

2.0 PURPOSE

The purpose of disinfectant Efficacy Study protocol is to ensure that the validated Disinfectants, concentration and contact time are effective against the environmental monitoring isolates by Tube Dilution method.

3.0 SCOPE

The Disinfectant Efficacy study protocol is applicable for Environmental monitoring isolates which are recovered as part of Environmental monitoring in Pharma Micro Hub Pvt Ltd.

4.0 REASON FOR STUDY

The reason for Preparation of this document is:

Please select any one (or multiple) option(s) from the following (☑)

- New Process ☐
- Changes / modification in area ☐
- Others (specify) _____ ☐
- Change Control details (If any):

5.0 ABBREVIATIONS

CAPA	: Corrective And Preventive Action
CGMP	: Current Good Manufacturing Practices
CFT	: Cross Functional Team
CFU	: Colony Forming Unit
LAF	: Laminar Air Flow Unit
MIS	: Miscellaneous
NA	: Not Applicable
NMT	: Not More Than
NLT	: Not Less Than
QA	: Quality Assurance
SOP	: Standard Operating Procedure

6.0 OVERVIEW

6.1.1 Efficacy of the disinfectants evaluated for compendial microorganisms and in-house isolates at different contact times for each concentrations of disinfectants in tube dilution method. Efficacy of validated disinfectant concentration in tube dilution study shall be evaluated on

different surfaces for validated contact time in surface challenge study. Most predominant surfaces selected for study, where the particular disinfectant is routinely used.

6.1.2 Disinfectant efficacy study was performed using compendial microorganisms and in-house isolates as per protocol. Concentration and contact times were established.

6.1.3 Hence Disinfectant efficacy study for in-house environmental isolates shall be performed only at established concentration of the disinfectant and contact times.

6.1.4 Disinfectant efficacy study shall be performed for new environmental isolate (New Species) by tube dilution method

6.1.5 The validation method mainly consists of the following phases.

- Preparation of challenge inoculum for the disinfectant efficacy study.
- Preparation of disinfectants as per SOP.
- Disinfectant efficacy study by Tube Dilution method.

7.0 RESPONSIBILITY

7.1 Microbiology:

- Preparation of protocol and report.
- Execution of activity as per protocol.

7.2 Quality Assurance

- Review and approval of protocol and report.

8.0 STUDY DESIGN

S. No.	Study Approach
1	The Disinfectant efficacy study designed by considering the following 1. To demonstrate the validated disinfectant concentrations as mentioned in Table-I are effective against environmental monitoring isolates. 2. To demonstrate the validated disinfectant contact time and hold period as mentioned in Table-I is adequate against environmental monitoring isolates. 3. Preparation of validated disinfectants (Validated Concentrations) as per SOP and holding of prepared disinfectant solutions as per validated hold period of 72 hrs and study shall be performed after hold period of 72 hrs. 4. Disinfectant efficacy study shall be performed for new environmental isolate (New isolates) by tube dilution method

9.0 TESTS, ITS RATIONALE AND ACCEPTANCE CRITERIA

The Disinfectant efficacy study with rationale and acceptance criteria are mentioned in the below table.

S. No.	Name/Title of test	Rationale	Acceptance criteria
1	Disinfectant Efficacy study for New Environmental monitoring isolates by Tube Dilution Method	➢ Disinfectant Efficacy study shall be performed for new environmental isolates (New Species). ➢ Disinfectant Efficacy study shall be performed as per USP<1072> Disinfectants and Antiseptics	➢ 2 log reduction for Bacterial and Fungal spores 3 log reduction for Vegetative microorganisms.

10.0 DISINFECTANT EFICACY STUDY PROCEDURE & OBSERVATIONS

10.1 EQUIPMENT/ MATERIALS REQUIRED FOR DISINFECTANT EFICACY STUDY:

Material Requirements:

10.1.1 Sterile Normal saline.

10.1.2 Sterile test tubes.

10.1.3 Sterile 0.1% peptone.

10.1.4 Ready to use Soybean-Casein Digest Agar petri plates (SCDA) with Neutralizer (0.5% tween 80 and 0.05% soya lecithin)

10.1.5 Sabourauds Dextrose Agar (SDA)

10.1.6 Sterile pipettes.

10.1.7 Sterile forceps.

10.1.8 Sterile membrane filtration units.

10.1.9 Sterile empty petri plates.

10.1.10 Sterile 0.45 µm PVDF Membranes.

10.1.11 Sterile funnel.

10.1.12 Sentino pump

10.1.13 Laminar air flow Unit

10.2 DISINFECTANT EFFICACY STUDY REQUIREMENTS AND OBSERVATIONS:

10.2.1 Selection of Disinfectants:

The following disinfectants were selected based on disinfectant efficacy study (Protocol No.: VP-MB-MIS-017**)**.

Table-I

Name of disinfectant	Approved manufacturer	Validated Testing concentrations	Validated Contact Time	Purpose of disinfectant
# Isopropyl alcohol	Any manufacturer	70% v/v	30 sec	General purpose disinfectant
Bacillocid (special)	Raman & weil pvt ltd	1.5%	15 Min	Sporicidal agent

Iso propyl alcohol disinfectant efficacy shall be challenge on only vegetative microorganisms.

10.2.2 Preparation of Inoculum:

10.2.2.1 The Viable microorganisms used in the study must be not more than five passages.

10.2.2.2 Procedure for Sub-Culturing:

10.2.2.2.1 Bacteria, Bacterial spores, Yeast & Fungal spores: Using a sterile pipette, transfer each 0.1 mL of culture from the cryovial or Take loopful of culture from plate to 10 mL of Soybean-Casein Digest Broth (SCDM) and incubate (Bacteria, Bacterial spores and Yeast) at 30°C -35°C for 24 to 48 hours and incubate (Fungal spores) at 30°C -35°C for 5 days. Document the details in Annexure-I.

10.2.2.3 Preparation of Bacterial & Yeast Culture Suspension:

10.2.2.3.1 After completion Incubation, transfer 1 mL of culture to 9 mL of sterile saline solution (10^{-1}) and perform serial dilution up to 10^{-7}

10.2.2.3.2 Plate out the dilutions from 10^{-3} to 10^{-7} in duplicates using Soyabean casein digest agar.

Note: Plating can be done by pour plate method using 1 mL of above suspension or spread plate method by using 0.1 mL of above suspension.

10.2.2.3.3 Incubate the plates at 30-35°C for 18 – 72 hrs for bacteria and at 20-25°C for 3 to 5 days for yeast. Document the details in Annexure-I.

10.2.2.3.4 Select a suspension of 10^6 CFU/0.1 mL for disinfectant efficacy study.

10.2.2.4 Preparation of Bacterial & Fungal Spore Suspension:

10.2.2.4.1 After completion Incubation, transfer 1 mL of the culture from SCDM onto roux bottle containing Soyabean Casein Digest agar and Spread the culture by rotating the roux bottle and incubate at 30-35°C for 5 days.

10.2.2.4.2 After completion of incubation, Harvest the cultures of spore forming organisms by transferring the 10 mL of sterile saline into roux bottle and collect the spores from the surface.

10.2.2.4.3 Centrifuge the obtained culture at 12,000 rpm for 10 min in order to separate the vegetative cells from spores.

10.2.2.4.4 Remove the supernatant and Wash the pellet by resuspend the pellet with sterile saline solution and centrifuge at 10,000 rpm for 12 min and repeat the same for 2 times to get the pure spores and then resuspend the pellet with 1.5 mL of sterile saline solution (Solution-A).

10.2.2.4.5 Make serial dilutions by transfer 1 mL of culture from Solution - A to 9 mL of sterile saline solution (10^{-1}) and perform serial dilution up to 10^{-5}

10.2.2.4.6 Plate out the dilutions from 10^{-2} to 10^{-5} in duplicates using Soyabean casein digest agar.

Note: Plating can be done by pour plate method using 1 mL of above suspension or spread plate method by using 0.1 mL of above suspension.

10.2.2.4.7 Incubate the bacterial spore forming organism's plates at 30-35°C for 18 – 72 hrs and Fungal spore forming organism's plates at 20-25°C for 3 to 5 days. Document the details in Annexure-II.

10.2.2.4.8 Select a suspension of 10^4 CFU/0.1 mL for disinfectant efficacy study.

10.3 Determination of disinfectant efficacy study by Tube Dilution method:

10.3.1 Preparation of Disinfectant: Dilute the desired concentration of disinfectant with water for injection and filter through the sterile 0.22µ membrane filter and collect the filtered disinfectant into a sterile container. Disinfectant preparation and filtration shall be performed in the laminar air flow unit only.

10.3.2 Hold the disinfectant container in the laminar air flow unit and study shall be performed after 72 hours hold time.

10.3.3 Perform the activity of disinfectant efficacy study by using all the disinfectants mentioned in Table-I.

10.3.4 TEST CONTROL (B):

10.3.4.1 Inoculate 0.1mL containing 10^6 cfu of test organism (Vegetative microorganism) into the 10 mL of specified concentration of disinfectants.

10.3.4.2 In case of spore forming organisms inoculate 0.1mL containing 10^4 cfu into the 10 mL of specified concentration of disinfectants.

10.3.4.3 Assemble the sterile membrane filtration set under Bio safety cabinet. Pre-wet the membrane with 50mL of sterile 0.1% peptone water. Then, filter 1mL of sample through the 0.45µm sterile membrane filter after specified contact time as mentioned in Table-1,.

10.3.4.4 Rinse the filter with 3 X 100mL of 0.1% peptone water. Remove the membrane filter and place on the surface of the Soyabean casein digest agar plates supplemented with neutralizer (0.5% tween 80 and 0.05% soya lecithin).

10.3.4.5 Place the membrane such that there are no air bubbles between membrane and the agar surface.

10.3.4.6 Perform above steps for all environmental monitoring isolates using all the disinfectants (as listed in the Table -I).

10.3.4.7 Incubate all the bacterial plates at 30-35°C for NLT 3 days and fungal plates at 20-25°C for NLT 5 days and document the analysis details in Annexure-III.

Note: In case of overgrowth observed in test control, then re-perform with serial dilutions up to 10^{-4} to find out the exact log reduction.

10.3.5 POSITIVE CONTROL (A):

10.3.5.1 Inoculate 0.1mL containing 10^6 cfu of test organism (Vegetative microorganism) into the 10 mL of 0.9% sterile normal saline.

10.3.5.2 In case of spore forming organisms, inoculate 0.1mL containing 10^4 cfu into the 10 mL of 0.9% sterile normal saline.

10.3.5.3 Perform serial dilution with 0.9% sterile Saline up to 10^{-6} for bacteria & yeast and 10^{-4} for spore forming organisms and filter 1mL from each dilution as below:

10.3.5.4 Assemble the sterile membrane filtration set under Bio safety cabinet (BSC). Pre-wet the membrane with 50mL of sterile 0.1% peptone water. Then, filter the 1 mL through the 0.45µm sterile membrane filter.

10.3.5.5 Rinse the filter with 3 X 100mL of 0.1% Peptone water. Remove the membrane filter and place on the surface of Soyabean casein digest agar plates supplemented with neutralizer (0.5% tween 80 and 0.05% soya lecithin).

10.3.5.6 Place the membrane filter such that there are no air bubbles between membrane and the agar surface.

10.3.5.7 Incubate all the bacterial plates at 30-35°C for NMT 3 days and fungal plates at 20-25°C for NMT 5 days and document the analysis details in Annexure-III.

10.3.6 NEGATIVE CONTROL:

10.3.6.1 Assemble the sterile membrane filtration set under Bio safety cabinet (BSC). Pre-wet the membrane with 50mL of sterile 0.1% peptone water. Then, filter the 1 mL of 0.9% sterile normal saline through the 0.45µm sterile membrane filter.

10.3.6.2 Rinse the filter with 3 X 100mL of 0.1% Peptone water. Remove the membrane filter and place on the surface of Soyabean casein digest agar plates supplemented with neutralizer (0.5% tween 80 and 0.05% soya lecithin).

10.3.6.3 Place the membrane filter such that there are no air bubbles between membrane and the agar surface.

10.3.6.4 Incubate all the bacterial plates at 30-35°C for NLT 3 days and fungal plates at 20-25°C for NLT 5 days and document the analysis details in Annexure-III.

10.3.7 Acceptance Criteria:

10.3.7.1 2 log reduction for bacterial and fungal spores and 3 log reduction for vegetative microorganisms within a recommended contact time.

10.3.7.2 Negative control should not show growth.

10.3.8 Interpretation of the results

If the log reduction met the acceptance criteria, the concentration of the disinfectant used are effective.

Invalidate the test, if the inoculum concentration falls less than 10^5 cfu / 1 mL of disinfectant solution. In the event of spore forming organism's inoculum concentration falls less than 10^3cfu / 1 mL of disinfectant solution.

11.0 REVALIDATION CRITERIA

Revalidation shall be carried out in below cases:

- Decrease/ Increase in disinfectant concentration
- Change in disinfectant formulation.
- Introduction of any new disinfectants.

12.0 ANNEXURES

Annexure-I : Report for Preparation of Bacterial & Yeast Suspension

Annexure-II : Report for Preparation of Bacterial & Fungal Spore Suspension

Annexure-III : Report for Determination of Disinfectant Efficacy study by Tube Dilution Method

Annexure -1

1. Sub culturing Details:

Name of the organism		Culture Suspension Reference No.:	
Date of sub culturing		Date of observation	
Name of the media & Lot number		Use before Date	
Cryovial ID		Valid up to	
Incubator ID No.:		Incubation condition	
Incubation Start Date & Time		Incubation End Date & Time	
Transfer _____ (0.1 mL) of culture from the cryovial or Take _____(loopful) of culture from plate to _____(10 mL) of Soybean-Casein Digest Broth (SCDM) and incubate at 30°C -35°C for 24 to 48 hours or 5 days		**OBSERVATION**	
		Growth observed / not observed	
Done by:		**Observed by:**	

2. Culture Suspension Details:

Date of Serial Dilution		Date of release	
Media used		Lot No	
Diluent used		Lot Number	
Incubator ID No.:		Incubation Condition	
Incubation Start Date & Time		Incubation End Date & Time	
Analyzed by:			

Dilution level	**Plate count CFU**		**Average CFU**	**Selected dilution for Disinfectant Efficacy study**
	Plate-1	**Plate-2**		
10^{-1}				
10^{-2}				
10^{-3}				
10^{-4}				
10^{-5}				
10^{-6}				
10^{-7}				

Remarks:

Observed by:		**Reviewed by:**	

Annexure -2

1. Sub culturing Details:

Name of the organism		Culture Suspension Reference No.:	
Date of sub culturing		Date of observation	
Cryovial ID		Valid up to	
Name of the media & Lot number		Use before date	
Incubator ID No.:		Incubation condition	
Incubation Start Date & Time		Incubation End Date & Time	
Bacteria, yeast, Bacterial spore & fungal spore culture Suspension: Transfer _____ (0.1 mL) of culture from the cryovial or Take _____(loopful) of culture from plate to _____(10 mL) of Soybean-Casein Digest Broth (SCDM) and incubate at 30°C -35°C for 24 to 48 hours or 5 days		**OBSERVATION**	
		Growth observed / not observed	
Done by:		**Observed by:**	

2. **Harvesting Details:**

Name of the media (Roux bottle) & Lot number	Soyabean Casein Digest Agar	**Use before date**	
Incubator ID No.:		**Incubation condition**	30 - 35°C
Incubation Start Date & Time		**Incubation End Date & Time**	
Analyzed by		**Observed by**	
Transfer _____(1 mL) of the culture from SCDM onto roux bottle containing Soyabean Casein Digest agar and Spread the culture by rotating the roux bottle and incubate at _____°C (30 - 35°C) for 5 days.		**OBSERVATION**	
		Growth observed / not observed	
Harvest the cultures of spore forming organisms by transferring the _____(10 mL) of sterile saline into roux bottle and collect the spores from the surface.			
Centrifuge RPM	10,000 rpm	**Centrifuge Time (10Min)**	From _____to_____
Pellet Washing			
1. Centrifuge RPM	10,000 rpm	**Centrifuge Time (12 Min)**	From _____to
2. Centrifuge RPM	10,000 rpm	**Centrifuge Time (12 Min)**	From _____to_____
3. Centrifuge RPM	10,000 rpm	**Centrifuge Time (12 Min)**	From _____to_____
Water bath ID No:		**Time of Heat shock**	From _____to_____

3. Culture Suspension Details:

Date of Serial Dilution		Date of Release	
Media used		Lot No	
Diluent used		Lot Number	
Incubator ID No.:			
Incubation Start Date & Time		Incubation End Date & Time	
Analyzed by: Sign & Date			

Dilution level	**Plate count CFU**		**Average CFU**	**Selected dilution for Disinfectant Efficacy study**
	Plate-1	**Plate-2**		
10^{-1}				
10^{-2}				
10^{-3}				
10^{-4}				
10^{-5}				
10^{-6}				
10^{-7}				

Remarks:

Observed by:		**Reviewed by:**	

Annexure -3

Date of Test:		Name of the Organism	
Culture Suspension Reference No.		Valid up to	
SCDA Lot No.		Use before Date	
0.1% Peptone Lot No.		Use before Date	
0.9% Saline Lot No.		Use before Date	
Filter cups Lot No.		Exp. Date	

Name of the Control	**Incubator ID**	**Incubation Date & Time**	**Incubation End Date & Time**
Positive control			
Test Control & Negative Control			

POSITIVE CONTROL (A):

Inoculum size	**Dilutions**					**Calculated count**	Log value of Calculated Count in Positive Control (A)	**Observed by Sign & Date**
	10^{-2}	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**			

TEST CONTROL (B):

S. No.	**Name of the Disinfectant**	**Concentration**	**Disinfectant preparation Date**	**Validated contact time**	**Recovery**					**Calculated count**	Log value of Calculated Count in Test Control (B)
					Direct sample (1 mL)	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**		
1	Bacillocid	1.5%									
2	Isopropyl alcohol	70%									
Negative Control											

LOG REDUCTION CALCULATION:

Name of the organism	Name of the Disinfectant	Validated Concentration	Log value of Calculated Count in Positive	Log value of Calculated Count in Test Control	**Log Reduction (A-B)**
	Bacillocid (Special)	1.5%			
	Isopropyl alcohol	70%			
Observed by:			Checked by:		

13.0 SUMMARY & CONCLUSION:

- Disinfectant efficacy study by tube dilution method has been performed as per protocol
- Disinfectant efficacy study has been performed for newly identified EM Isolate-*micro coccus luteus*
- with validated disinfectants, concentrations, 72 hrs hold time and contact time of Bacillocid special-1.5% and IPA- 70%.
- EM Isolate-*Staphylococcus epidermidis* Log reduction (4.9) found more than 4 log reduction, which is meeting the acceptance criteria of 3 log reduction.
- Based on the above summary, it is concluded that the validated disinfectant concentrations are very effective and disinfectants hold time of 72 hrs and contact time is adequate against new environmental Isolate- *micro coccus luteus.*

14.0 POST-APPROVAL

Signing of this approval page of report indicates agreement with the validation approach described in this document.

Name	Department/Designation	Signature	Date
Prepared By:			
Reviewed By:			
Approved By:			