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ENUMERATION AND ISOLATION OF MICROBIAL CONTAMINANTS THROUGH ENVIRONMENTAL MONITORING

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ABSTRACT

The monitoring is done to check the bioburden of the aseptic area of the controlled environment. The purpose of this is to understand the various issues that is related to aseptic processing of bulk drug substance or finished products (sterile), dose forms, and in certain cases and to establishments maintenance and control of the microbiological quantity in the controlled environment. Environmental monitoring describes the processes and activities that need to take place to characterise and monitor the quality of the environment. Environmental monitoring is used in the preparation of environmental impact assessments, as well as in many circumstances in which human activities carry a risk of harmful effects on the natural environment. All monitoring strategies and programmes have reasons and justifications which are often designed to establish the current status of an environment or to establish trends in environmental parameters. In all cases the results of monitoring will be reviewed, analysed statistically, and published. The design of a monitoring programme must therefore have regard to the final use of the data before monitoring starts. The number of viable microorganisms detected during routine monitoring may be within the relevant acceptance limits. However, it is necessary to characterize and identify these microorganisms to their genus and species level. Also, and identification of different types of microbial isolates found during routine monitoring is required to check whether the microorganisms obtained are not objectionable types. Objectionable types microorganisms are the ones which may be detrimental to the product or the process or pathogenic in nature.

KEYWORDS: Microorganisms, Isolation of microbes, Environmental monitoring

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INTRODUCTION

Monitoring is performed to verify the bioburden of the regulated environment's aseptic region. The aim of this is to explain the different issues relating to the aseptic processing of bulk drug substances or finished (sterile) products, the dosage forms and, in some cases, the conservation and monitoring of microbiological quantities in the regulated environment by the facilities [1]. Estimating the amount of viable aerobic micro-organisms present in the atmosphere by monitoring of the atmosphere. Monitoring is performed to verify the bioburden of the regulated environment's aseptic region. The aim of this is to explain the different issues relating to the aseptic processing of bulk drug substances or finished (sterile) products, the dosage forms and, in some cases, the conservation and monitoring of microbiological quantities in the regulated environment by the facilities [2].

In order to characterise and track the quality of the atmosphere, environmental management defines the procedures and activities that need to take

place. Environmental monitoring is used in the preparation of environmental impact assessments, as well as in certain cases where there is a chance of adverse effects on the natural environment from human activities. There are explanations and justifications for all monitoring techniques and programmes that are mostly structured to assess the current state of the environment or to determine patterns in environmental parameters. The monitoring results will be checked, statistically evaluated and reported in all cases. Therefore, the design of the monitoring software must take into account the final use of the data before monitoring begins [3].

MATERIALS AND METHODS

Air sampling is a compulsory process to be practised in pharmaceuticals to produce a reliable product free of contamination. Another main source of pollution, however, is air, which most pharmaceutical facilities frequently neglect to take precautions. For quality control (QC) purposes, air particulate sampling or air monitoring is important , especially in companies that produce pharmaceutical products in regulated areas or clean rooms with filtered air.

Well, what pharmacists, health care professionals, and microbiologists refer to as air sampling can simply be defined as the correct measurement of realistic airborne bacteria , mould, yeast, spores, and fungal cells that can be collectively referred to in the surrounding air as bioburden and is intended to track the environment [4]. Petri dishes, air sampler, sampler for compressed air, medium nutrient agar, incubators, SDA, SCD Laminar Air Flow.

Procedure & Frequency of Testing Under

1. Control by settle plate counts (Passive Air Monitoring)

Preparations

- ✓ Prepare required quantity of soyabean digest agar medium and sterilize the medium in an autoclave at 121 °C for validate time setting.
- ✓ Aseptically pour about 20ml of molten sterilized soyabean casein digest agar medium previously cooled to not more than 50 ºC into dry sterilized 90mm petri dishes. Cover the lids of petri dishes and allow the medium to solidify under the Laminar Air Flow bench.
- ✓ Incubate all the Petri dishes at 30ºC to 35 ºC for 36-48 hours. This is pre-incubation.
- ✓ Use petri dishes with absolutely no sign of microbial contamination at end of incubation for subsequent exposure.
- ✓ The petri dishes shall be serially numbered along with date of exposure. In order to avoid mix up of petri dishes of one area with those of other area, code no should be assigned indicating the location under microbiological monitoring. The petri dishes shall also indicate an abbreviation for the test for which the same are used.
- ✓ The abbreviations SPC, CPC and VAS shall be used to indicate the petri dishes used for settle plate counts, contact plate and volumetric air sample counts. Similarly, the abbreviations for various departments shall be as follows: External Preparation=E, Oral hygiene=O, Warehouse=W, Microbiology=M and Liquid=L [5].

Sampling and Observations

- ✓ Load the Petri dishes with no visible sign of contamination in the clean and disinfected S.S. Canister. close and carry the canister to the respective area.
- ✓ Enter the area under monitoring after strictly following the appropriate entry procedure. Ensure that the area under monitoring is cleaned and sanitized as per routeing procedure. In order to have a meaningful outcome of such monitoring, the microbiological monitoring of environment shall be performed under normally prevailing conditions and not under extra ordinary cleaned and sanitized environment.
- ✓ Ensure that the temperature/humidity in the area under microbiological monitoring is within the specified limits.
- ✓ Place the Petri dishes containing soyabean casein digest agar medium as prepared above carefully number wise, at the designated places as shown in the schematic exposure chart, prepared for the relevant area under monitoring except for one petri-dish containing soyabean casein digest agar medium “DO NOT EXPOSE”. This is negative control.

- ✓ Clean the SS stand with 70% IPA. Place the petri dish over the SS stand having height of 22 inches and carefully lift the lid of each petri dish.
- ✓ Leave the petri dishes in the exposed condition for 1 hour and leave the area.
- ✓ Re- enter the area after 1hour (specified tome at the end of exposure period as the case may be).
- ✓ Gently place the lids over the exposed petri-dishes and load them back in the Canister, bring them out of the controlled area.
- ✓ Incubate all the exposed petri-dishes in an inverted position at 20°C to 25°C for three days followed by two days at 30 °C to 35°C. Daily during the incubation period, petri dishes will be observed in order to get an advanced alert in the event of adverse observations.
- ✓ Count and record the number no. of colony forming units developed on each of the petri dishes at the end of incubation of five days.
- ✓ Successful demonstration of compliance to the acceptance criteria with respect to growth promotion test during media evaluation test on SCD [6].

2. Surface controls by Contact Plate Counts
Preparations

- ✓ RODAC plates are used for determination of surface counts. These are 55mm diameter empty plates and are available in a sterilized form in poly packs. The nutritive medium is filled later
- ✓ Preparation requires quantity of Soyabean casein digest agar medium containing 4% v/v polysorbate 20 and 0.5% w/v soya lecithin defined procedures. Sterilize the medium in an autoclave 121 ºC for validated time setting.
- ✓ Under strict aseptic conditions open the plastic bag containing sterilized disposable plates, place the plates on the Laminar Air Flow bench and carefully remove the lids of the plates.
- ✓ Aseptically pour sufficient quantity of molten sterilized Soyabean Casein Digest Agar medium to containing 4% v/v polysorbate 20 and 0.5% w/v soya lecithin in previously cooled to not more than 50 °C to each of the plate in such a way that the level of the medium in the plate is just higher than the edge of the plate. Regulate the quantity of the medium carefully to avoid the overflow of the same. Allow the medium to solidify then replace the lids of the contacts plates.
- ✓ Incubate all the plates at 30ºC to 35ºC for 36-48 hours. This is pre-incubation. Use only those plates showing absolutely no sign of contamination for subsequent testing [7].

Sampling and Observations

- ✓ Load the plates with no contamination in a clean and disinfected S.S. Canister. Close and carry the canister for the exposure to the respective department.
- ✓ Enter the area under monitoring after strictly following the written entry procedures.
- ✓ Product contact surface shall not be monitored using contact plates in order to prevent contamination due to residue of culture medium.
- ✓ While using a contact plate for surface monitoring in case if it us noticed that the rim of contact plate touches the surface to be monitored, it is an indication that the level of the medium is not raised. Such contact shall not be used for surface monitoring.
- ✓ In order to take the sample for surface count by contact plate, remove the lid of the plate, lay the side of the plate containing the nutritive medium facing downwards on the surface to be examined and press it gently. Leave one contact plate in opened in the canister with the marking “DO NOT EXPOSE” on it. This is negative control (The location code for various Departments shall be as follows: External Preparation=E, Oral Hygiene=O, Warehouse=W, Microbiology=M and Liquid=L).
- ✓ Lift off plate without sliding along the surface and replace the lid gently.
- ✓ After taking the sample from the contact surface, wipe the place thoroughly with 70% v/v IPA to remove any residual traces of the nutritive medium and disinfect the affected surface.
- ✓ Repeat the sampling procedures as above at all the sites as specified for the purpose.

- ✓ Incubate the contact plates at 20ºC to 25 ºC for three days followed by two days at 30ºC to 35ºC.
- ✓ Count and record the number of CFUs developed on the contact plates at the end of five days incubation. This indicates the no. of viable microorganisms per 55mm diameter contact plate.
- ✓ Successful demonstration of compliance to the acceptance criteria with respect to growth promotion test during media evaluation test on SCD agar [6].

3. Control by volumetric Air Sampling
Preparation

- ✓ Prepare required quantity of Soyabean Casein Digest agar medium, sterilized the medium in an autoclave at 121 °C for validated time setting.
- ✓ Aseptically pour about 20 ml of molten sterilized Soyabean Casein Digest Agar medium previously cooled mot more than 500C into dry sterilized 90 mm petri-dishes. Cover the lids of petri-dishes and allow the medium to solidify under the Laminar Air Flow bench,
- ✓ Incubate all the petri-dishes at 30 ºC to 35 ºC for 36-48 hours. This is Pre- incubation.
- ✓ Use petri-dishes with absolutely no sign of microbial contamination at the end of incubation for subsequent exposure. This is to ensure sterility of the culture medium used for Volumetric Air Sample counts. Mark the petri-dishes as per the procedures described above [8].

Sampling and Observation

- ❖ Sterilize the perforated lid and the dust cover of the air sampler in an autoclave at 121ºC for validated time setting.
- ❖ Incubate all the petri-dishes for three days at 20º C to 25ºC followed by two days at 30ºC to 35ºC. petri-dishes will be observed daily in order to get an advanced alert for an unusual observation. However, the recording of observation shall be done at the end of incubation.
- ❖ At the end of incubation of five days count and record the number of colony forming unit developed on each of the petri-dishes. The total number of CFUs is corrected using Fellers Statistical Correction Table and the corrected value is treated to get number of CFUs per cubic meter of air (1000 litres)

4. By Finger Dab Method Environment monitoring (or Microbial Monitoring of Hand Gloves of Personal Working in Core Area)
Procedure

Microbiological Monitoring of hand gloves persons shall be performed by Finger Dab method.

Preparation

- ✓ Prepare required quantity of Soyabean Casein Digest Agar containing Soya Lecithin Medium and dispense required quantities in conical flasks. Sterile the medium in an autoclave at 121 °C for validated time setting.
- ✓ Under strict aseptic conditions open the butter paper containing sterilized glass plates, place the plates on the Laminar Air Flow bench and carefully remove the lids of plates.
- ✓ Aseptically pour sufficient quantity of molten sterilized Soyabean Casein Digest Agar containing soya Lecithin Agar Medium previously cooled to not more than 50 °C to each of the plate. Replace the lids of plates and allow the medium to solidify.
- ✓ Incubate all the plates at 30 °C to 35 °C for 48 hours. This is pre- incubation. Use only those plates showing absolutely no sign of microbial contamination for subsequent testing [9].

Sampling Location

- ❖ Sampling carried out on personal working in,
- ❖ Warehouse Area; Dispensing-1 Dispensing-2, sampling-1, sampling 2 Tablet area, oral liquid sampling and dispensing area.
- ❖ Microbiology section: Testing room persons who perform the test.
- ❖ Tablet section: Coating-1, Coating-2, Granulation-1, Granulation-2, Compression-1 to 4 and Packing-1 to 5.
- ❖ Oral Hygiene, External preparation and Oral liquid section- Filling area and

manufacturing area persons are monitored for finger dab.

Sampling

- Load the plates with no contamination in a clean and disinfected S.S. canister. Close and carry the drum for the exposure to the respective department.
- Enter the area under monitoring after strictly following the written entry procedures.
- The hand gloves shall not be monitored immediately after regular disinfection. The same shall be monitored just prior to regular disinfection in order to sample the "worst case".
- Aseptically open the plate in the core area and ask to the person working in that particular area to press his\her fingers followed by the thumb on the media plate. Use one single plate for single hand.
- Close the plate with lid and mark the following details on the base of plate; Name of person, working location, and date of sampling
- Disinfect both hands and fingers of the person thoroughly with 70% IPA to remove any residual traces of the nutritive medium and then discard the hand gloves.
- Repeat the sampling procedure as above for other personal working in the core area.
- Incubate the plates at 20 °C to 25 °C for three days followed by two days at 30 °C to 35 °C.
- Count and record the number of CFUs developed on the plates at the end of five days incubation. This indicates the number viable microorganisms as 5 fingers with gloves [10].

Acceptance Criteria

- Alert limit is 15 CFU/5 fingers with gloves and Action limit 20 CFU/5 fingers with gloves. Limits shall be established on the basis of trend data.
- The Positive control Test shall be the compliance of the SCDA containing soya lecithin medium for GPT.
- The Negative Control Test must not reveal any Colony Forming units of the preincubated plates for monitoring of hand gloves.

Identification of Isolates

- ✓ Procedure for isolation, characterization and identification of microbial isolates and confirmatory tests of specified microorganisms.
- ✓ The number of viable microorganisms detected during routine monitoring may be within the relevant acceptance limits. However, it is necessary to characterize and identify these microorganisms to their genus and species level. Also, and identification of different types of microbial isolates found during routine monitoring is required to check whether the microorganisms obtained are not objectionable types. Objectionable types microorganisms are the ones which may be detrimental to the product or the process or pathogenic in nature [11].

Morphological Characteristics

The morphological characteristics of the term relate to the identifiable characteristics of an agar plate colony. Colonies that vary in appearance are usually strains, species, or genera of different organisms. The classification of morphological features includes the form of the colony, the margins or edges of the colony, the colour of the colony and its surface characteristics.

These morphological features and the macroscopic characteristics of the agar plate microbial isolate can be used to aid the identification process [12]. Observe the description of the colonies with respect to the morphological characteristics given below after completion of incubation:

Size

Measure the diameter of the colony approximately in mm and report the diameter in ranges of as given below:

a) <1 mm to 1mm

b) 1 mm to 3 mm

c) 3 mm to 6 mm

d) 6 mm and above

Shape/Form

Report the shape/ Form as follows:

a) Circular- in the shape of circle.

b) Filamentous- the shape of colony is circular with margin ending with hair like structures.

c) Irregular- un- uniform shape with margin having lobe like structures.

d) Rhizoid- the shape with margin of branching root like structures.

e) Spindle- is oval shape colony.

f) Punctiform- minute (dot) like colonies where is <1 mm to 1mm.

Colour

Report the colour of colony as follows:

a) Cream

b) Yellow

c) Orange

d) Pink

e) Red

f) White

g) Black

h) Brown

Margin

Report the margin of colony as follows:

a) Entire – the edge of the colony is smooth and circular.

b) Undulate – the edge of colony is irregular.

c) Lobate – the colony with lobe like margin.

d) Erose – the colony with spiky margin.

e) Filamentous – the edge of colony is thread like structures

f) Curled – the edge of colony is concentric curled circles.

Elevation

Repot the elevation parameter with respect to the shape above the surface of the medium as follows:

a) Flat- the colony almost in level with the surface of the medium.

b) Raised – slightly raised above the surface of the medium but flat.

c) Convex – slightly raised above the surface of the medium in curve shape.

d) Pulvinate – raised above the surface of the medium in curve shape (less distinct).

e) Umbonate – look like raised colonies but they have a small projection in the middle (more distinct)

Consistency

Report the consistency of colony as follows:

a) Butyrous – butter like

b) Mucoid – slimy

c) Dry

Isolation of Colonies

A portion of the different morphological colonies is individually transferred aseptically in tubes each containing 2-3 ml of saline. These colonies may form a nutrient medium surface found in Petri dishes, as in the case of environmental monitoring, water sampling, product testing, or may form a membrane philtre surface, as in the case of water isolates, product bioburden, etc. Gently shake the tubes of normal saline to homogenize the contents.

Aseptically streak the above suspension loop onto the Casein Soyabean Digest Agar or R2A agar surface found in a Petri-dish to get isolated colonies, invert the dish and incubate for 24-72 hours at 30 to 35 °C.

Observe the CFUs developed on the Petri dishes critically to ensure that there is no evidence of microbial contamination and all the isolates colonies are of similar appearance [13].

Aseptically move a portion of a well-isolated, well-developed colony individually to three slants containing casein soyabean digest agar or R2A agar, incubate all slants for 24-72 hours at 30 to 35 °C. For further biochemical characterization, one slant containing pure isolates shall be used, the second slant shall be used for the identification of the microorganism by means of the BBL crystal technique, and the third slant shall be maintained at 2-8 °C for the maintenance of culture.

Gram Staining

Gram Staining is a technique of differential staining which distinguishes bacteria into two main classes, gramme positive and gramme negative. The technique is based on the distinction between the composition of various bacteria in the cell wall. A higher lipid content or

protein content is found in certain bacterial cell walls. Even, for these elements, the stains used in gramme staining have different affinities and they bind with them reversibly or irreversibly. Gram-positive bacteria thus irreversibly bind the stain and cannot be decolorized by alcohol, whereas gram-negative bacteria reversibly bind the stain and give it away when alcohol is processed. They then pick up the secondary stain and become stained pink [14].

Biochemical Characterization

- ✓ Prepare the inoculum by picking up 2 loopfuls of pure culture from casein soyabean digest agar or R2A agar slant meant for biochemical characterization and making a homogenous suspension in 3ml sterile saline.
- ✓ The Hi carbonate kit contains following Part A, Part B each having 12 carbohydrates utilization tests and Part C containing 11 sugars and 1 controlOpen the kit aseptically and peel off the sealing foil.
- ✓ Inoculate each well with 50µl of the inoculum by surface inoculation method.
- ✓ Incubate the kit at 3C to 38 °C for 18- 24 hours.
- ✓ At the end of incubation observe the reactions on the test kit and interpret the observation as given in biochemical characterization interpretation chart.
- ✓ After use, kits and the contaminated articles used for isolation and inoculation shall be handled, described "Procedure for decontamination, disposal of decontaminated media and washing of glassware's and accessories".

Identification of Microbial isolates

The indication of microbial isolates to their genus and species level shall be carried out using BBL Crystal identification system.

Gram Positive ID Kit (For Identification of Gram-Positive Bacteria)

A miniaturised identification method employing modified traditional, fluorogenic substrates is the BBL Crystal Gram Positive (GP) Identification (ID) system. It is designed to recognise frequently isolated Gram-positive aerobic bacteria. 29 dried biochemical and enzymatic substrates include the BBL Crystal GP ID panels. For re-hydration of substrates, a bacterial suspension in the inoculum fluid is used. The tests used in the system are based on microbial utilization and degradation of specific substrates detected by various indicator systems.

Fluorogenic substrate enzymatic hydrolysis results in increased fluorescence that can be visually observed under UV light. On hydrolysis, chromogenic substrates produce colour changes that can be visually observed. There are also tests that detect an organism's ability to hydrolyse, degrade, minimise or otherwise use a substrate ID package.

Enteric-Non-Fermenter ID Kit (For Identification of Gram-Negative Bacteria)

The BBL crystal E / NF ID system is designed to recognise aerobic Gram-negative bacteria belonging to the Enterobacteriaceae family, as well as some of the commonly isolated Gram-negative Bacilli glucose fermenting and non-fermenting bacteria. It is a miniaturised identification device that involves fermentation, oxidation, degradation and hydrolysis tests of different substrates. In addition, there are chromogen linked substrates to detect enzymes that microbes use to metabolize various substrates[15].

Identification of Gram-Positive Organism

- ✓ Use BBL crystal G.P. ID system for identification of gram-positive organisms.
- ✓ Remove the lids from pouch. Discard desiccant. Once removed from the pouch, covered lids shall be used within one hour. Do not use the panel if there is no desiccant in the pouch.
- ✓ Take an inoculum fluid tube and label it with the details about the isolate under identification.
- ✓ Using a sterilizes wireloop, aseptically pick a portion a portion of well isolated colony grown on slant of Casein Soyabean Digest agar medium or R2A agar medium.
- ✓ Aseptically suspended the picked portion of colony into a tube containing inoculums fluid.
- ✓ Recap the tube containing inoculum fluid and vortex mix the suspension for 10 to 15 seconds.
- ✓ The turbidity of the suspension should be equivalent to a McFarland No. 0.5

standard. In case the inoculum suspension has turbidity higher than the recommended McFarland standard, discard the same and prepared a fresh inoculum suspension.

- ✓ Take a base and write the details about the culture under identification on the side wall.
- ✓ Pour the entire contents of the inoculum fluid tube into target area of the base.
- ✓ Hold the base in both the hands and roll inoculum gently along the tracks until all of the wells are filled. Roll back any excess fluid to the target area and place the base on a bench top.
- ✓ Align the lid so that the labelled end of the lid is on top of the target area of the base.
- ✓ Push down until a slight resistance is felt, Place thumb on edge of lid towards middle of panel on each side and push downwards simultaneously until the lid snaps into place (listen for two "clicks").
- ✓ The inoculated panel shall be incubated face down at 350c to 370c for 18 to 24 hours. In case the panel is incubated foe 24 hours, the same shall be read within 30 minutes after removing from incubator.
- ✓ Using a sterilized wireloop, aseptically remove a small drop from the inoculum fluid tube either before or after inoculating the base and streak over a surface of Soyabaean Casein Digest Agar medium, R2A agar medium contained in a petri dish. Invert and incubate this purity plate at 35 to 37 °C for 48 hours.
- ✓ At the end of incubation, remove the panel from the incubator, the panel shall be read face down using a BBL crystal panel viewer.
- ✓ BBL crystal operated with crystal MIND software which is installed on computer.
- ✓ With the help of mouse click "SCAN". After scanning click "ADD" and "ID" button displayed on icon.
- ✓ Click on "Review" displayed on icon. Then print out to obtain the identification results.

Identification of Gram-Negative Organisms

- ❖ Use BBL crystal Enteric/non-Fermenter ID kit for identification of gram-negative organisms.
- ❖ BBL Crystal E/F system requires oxidase test and indole test results for the isolates under identification. Perform these tests and isolated colonies of a freshly grown culture as per the procedure described below.
- ❖ Remove the lids from pouch. Discard desiccant. Once removed from the pouch, covered lids shall be used within one hour. Do not use the panel if there is no desiccant in the pouch.
- ❖ Take an inoculum fluid tube and label it with the details about the isolate under identification.
- ❖ Using a sterilizes wireloop, aseptically pick a portion a portion of well isolated colony grown on slant of Casein Soyabean Digest agar medium or R2A agar medium.
- ❖ Aseptically suspended the picked portion of colony into a tube containing inoculums fluid.
- ❖ Recap the tube containing inoculum fluid and vortex mix the suspension for 10 to 15 seconds.
- ❖ The turbidity of the suspension should be equivalent to a McFarland No. 0.5 standard. In case the inoculum suspension has turbidity higher than the recommended McFarland standard, discard the same and prepared a fresh inoculum suspension.
- ❖ Take a base and write the details about the culture under identification on the side wall.
- ❖ Pour the entire contents of the inoculum fluid tube into target area of the base.
- ❖ Hold the base in both the hands and roll inoculum gently along the tracks until all of the wells are filled. Roll back any excess fluid to the target area and place the base on a bench top.
- ❖ Align the lid so that the labelled end of the lid is on top of the target area of the base.
- ❖ Push down until a slight resistance is felt, Place thumb on edge of lid towards middle of panel on each side and push downwards simultaneously until the lid snaps into place (listen for two "clicks").
- ❖ The inoculated panel shall be incubated face down at 35 to 37 °C for 18 to 24 hours. In case the panel is incubated foe 24 hours, the same shall be read within 30 minutes after removing from incubator.
- ❖ Using a sterilized wireloop, aseptically remove a small drop from the inoculum fluid tube either before or after inoculating

the base and streak over a surface of Soyabaean Casein Digest Agar medium, R2A agar medium contained in a petri dish. Invert and incubate this purity plate at 35 to 37 °C for 48 hours.

- ❖ At the end of incubation, remove the panel from the incubator, the panel shall be read face down using a BBL crystal panel viewer.
- ❖ BBL crystal operated with crystal MIND software which is installed on computer.
- ❖ With the help of mouse click "SCAN". After scanning click "ADD" and "ID" button displayed on icon.
- ❖ Click on "Review" displayed on icon. Then print out to obtain the identification results [16].

Confirmatory and Identification Tests for Specified Micro-Organisms [17, 18]

Harmonized microbiological test protocols for the detection of specified micro-organisms include the performance of confirmatory and identification tests to assess with confidence the presence / absence of the specified micro-organism in the preparation under study.

These confirmatory and identification tests for each specified micro-organism shall be performed as per the procedure described below.

Confirmatory Test for *Escherichia coli*
Confirmatory Test

- The growth of colonies on MacConkey agar is an indication for probable presence of Escherichia coli in the preparation under examination.
- Observe the colony morphology of the organism grown on MacConkey agar and carry out the microscopic examination.
- Perform the gram staining on the portion of the colony grown on MacConkey agar and record.
- Perform the Indole test as follows:
- Aseptically transfer the suspect colony from MacConkey agar to a tube containing 10 ml peptone water, shake the tube to homogenize the contents and incubate at 43.5 to 44.5 °C for 24 hours. To test for indole, add 3ml peptone water tube and extract by shaking for one minute. Allow to get both the layers separated for two minutes and add 0.5 ml Kovacs reagent and allow to stand for one minute. Appearance of indole.
- The absence of red non mucoid colonies of gram-negative bacteria on MacConkey agar and/or a Negative Indole test confirms the absence of Escherichia coli in the preparation under examination.

Identification Test (To be Performed only if Confirmatory Tests are Positive)

- ❖ Identify the suspected isolate as per the procedure described above using BBL Crystal Enteric/non -Fermenter ID kit for identification of gram -negative organisms.
- ❖ The preparation under examination shall be considered to be free from *Escherichia coli* if the identity of the organism is revelled to be other than Escherichia coli.

Confirmatory Test for *Salmonella*
Confirmatory Test

- The growth of well developed, red colonies with colonies with or without black centres on the surface of Xylose lysine deoxycholate agar is an indication for probable presence of *Salmonella* in the preparation under examination.
- Observe the colony morphology of the organism grown on Xylose Lysine agar and carry out the microscopic examination.
- Perform the gram staining on the portion of the colony grown on Xylose lysein deoxycholate agar and record.
- Perform the test for H2S production as follows:
- Sub-culture apportions of the suspect colony matching with the description as above to the surface of triple sugar iron agar by first inoculating the surface of the slant and then making a stab culture with the same inoculating wire. Incubate the inoculated medium at 36 to 38 °C for 18-24 hours. The presence of Salmonella is provisionally confirmed if in the stab but not on the surface there is a change of color from red to yellow and usually a formation of gas with or without production of hydrogen sulphide indicated by blackening.
- The absence of well-developed and red colonies with or without black centres on the surface of Xylose lysein deoxycholate

agar of gram-negative bacteria and / or a Negative observation for H_2S production.

Identification Test (To be Performed only if Confirmatory Tests are Positive)

- Identify the suspected isolate as per the procedure described above using BBL Crystal Enteric/non- Fermenter ID kit for identification of test confirms the absence of *Salmonella* in preparation under examination.
- The preparation under examination shall be considered to be free from *Salmonella* if the identity of the organism is revelled to be other than salmonella.

Confirmatory Tests for *P. aeruginosa*
Confirmatory Test

- ❖ The growth of colonies on the surface of Cetrimide agar is an indication for probable presence of *Pseudomonas aeruginosa* in the preparation under examination.
- ❖ Observe the colony morphology of the organism grown on Cetrimide agar and carry out the microscopic examination.
- ❖ Perform the gram staining on the portion of the colony grown on Cetrimide agar and record the observations.

Perform the Elevated Temperature test as follows:

- Aseptically transfer some portion of morphologically different isolated colonies separately to each tube containing 10 ml Casein soyabean digest broth. Incubate the tubes at 41 to 43 °C for 18-24 hours. No visible evidence of microbial growth in the medium at the end of incubation confirms the absence of *P. aeruginosa* in the preparation under examination.

Perform the Oxidase Test as follows:

- On oxidase disc smear apportion of the colony of the organism under examination. In case there is no development of pink color, changing to purple, the suspect organism shall be termed as oxidase negative.
- The absence of colonies of gram-negative organisms on the surface of cetrimide agar and / or negative observation for elevated temperature test and / or a Negative oxidase test confirms the absence *of P. aeruginosa* in the preparation under examination.

Identification Test (To be Performed only if Confirmatory Tests are Positive)

- Identify the suspected isolate as per the procedure described above using BBL Crystal Enteric/non-Fermenter ID kit for identification of gram-negative organisms.
- The preparation under examination shall be considered to be free from P. aeruginosa if the identity of the organism is revelled to be other than *P. aeruginosa*.

Confirmatory Tests for *Staphylococcus aureus*
Confirmatory Test

- ❖ The growth of yellow/ white colonies surrounded by a yellow zone on the surface of Mannitol salt agar is an indication for probable presence of *Staphylococcus aureus* in the preparation under examination.
- ❖ Observe the colony morphology of the organism grown on Mannitol salt agar and carry out the microscopic examination.
- ❖ Perform the gram staining on the portion of the colony grown on mannitol salt agar and record the observations.

Perform the confirmatory test as follows:

- Aseptically streak a loopfull of suspension of suspect colony on surface of Baired – Parker agar contained in a Petridis, invert and incubate the petri dish at 30 to 35 °C for 18-24 hours. No development of black shiny colonies surrounded by black zones confirms the absence of *S. aureus* in the preparation under examination.
- The absence of yellow/white colonies surrounded by yellow zone of gram - positive organisms (cocci in clusters) and /or absence of black shiny colonies surrounded by black zones confirms the absence of *Staphylococcus aureus* in the preparation under examination
- The preparation under examination shall be free from *Staphylococcus aureus* if the identity of the organism is revealed to be other than *Staphylococcus aureus.*

Identification Test (To be Performed only if Confirmatory Tests are Positive)

- Identify the suspected isolate as per the procedure described above using BBL Crystal G.P ID system for identification of gram-positive organisms.
- The preparation under examination shall be considered to be free from *S. aureus* if the identity of the organism is revelled to be other than staphylococcus aureus.

Confirmatory Tests for *Shigella spp*
Confirmatory Test:

- ❖ The growth of red colonies without black centres on the surface of Xylose lysine Deoxycholate agar is an indication for probable presence of Shigella spp.in the preparation under examination.
- ❖ Observe the colony morphology of the organism grown on Xylose Lysine agar and carry out the microscopic examination.
- ❖ Perform the gram staining on the portion of the colony grown on Xylose lysein deoxycholate agar and record.

Perform the test for H_2S production as follows:

- Sub-culture apportions of the suspect colony matching with the description as above to the surface of triple sugar iron agar by first inoculating the surface of the slant and then making a stab culture with the same inoculating wire. Incubate the inoculated medium at 36 to 38 °C for 18-24 hours. The presence of Shigella is provisionally confirmed if in the deep inoculation there is change in color from red to yellow (acidic butt) and on the surface there is no change.
- The absence of well-developed and red colonies without black centers on the surface of Xylose lysein deoxycholate agar of gram-negative bacteria and / or a Negative observation for H_2S production test confirms the absence of Shigella in the preparation under examination.

Identification Test

- (To be performed only if Confirmatory tests are positive)
- Identify the suspected isolate as per the procedure described above using BBL Crystal Enteric/non-Fermenter ID kit for identification of Gram-negative organisms
- The preparation under examination shall be considered to be free from *Shigella* if the identity of the organism is revelled to be other than *Shigella*.

RESULTS

The results during these three months dissertation done by me in microbiological monitoring of environment by active air monitoring, passive air monitoring, contact plate count.

Table 1: Number of Colonies Found

Areas for Monitoring	Volumetric Air sampling or Active air sample (cfu/m^3)	Settle plate or passive air monitoring 90 mm (cfu/4- hour exposure)	Contact plate, 55 mm (cfu/plate)	Glove print, five fingers (cfu/glove)
Grade A (local zones for high-risk operation, e.g., point-of-fill, protection of aseptic connections, etc.)	<1	<1	<1	<1
Grade B (e.g., in the case of aseptic manufacture Grade B is the background environment for Grade A zones)	8 cfu	4 cfu	3 cfu	NA
Grade C (e.g., rooms where aseptic solutions are prepared for filtration)	97cfu	47 cfu	23 cfu	NA

The count was found to be less than 1CFU / m^3 by volumetric sampling in grade A, the CFU count was exceeded to 8 in grade B and increased to 97 CFU in grade C count. For passive air monitoring, the count was found to be less than 1 CFU / m^3 in grade A, the CFU count was exceeded to 4 in grade B, and increased to 47 CFU in grade C. The count was found to be less than 1 CFU / m^3 in grade A for contact plate monitoring, the CFU count was exceeded to 4 in grade B and increased to 47 CFU in grade C count. With less than 1 cfu / glove, the finger dab of the employees working in the grade A zone was found.

The colonies in the three grades do not contain any of the pathogenic strains mentioned above. *Kytococcus sedanterius, Micrococcus luteus, Micrococcus lylae* and *Staphylococcus aureus* are orange, purple, cream, white and circular in form with gramme-ve cocci, respectively, the colonies present in grade B and C.

Each of the pathogenic strains listed above does not include the colonies that were in three grades. The presence of these M.O was not found to be pathogenic or hazardous to the preparation of drugs, so the results of the work carried out above are that the drug manufacturing and processing area is sufficient.

Passive Air Sampling

- Exposure time – 4 hours TBC- 100 CFU/Plate
- TFC- 10 CFU/Plate

Table 2: Passive Air Sampling

Area	TBC	TFC
Changing Room	10 CFU/Plate	< 1
Crossing bench	25 CFU/Plate	<1
Media preparation room	20 CFU/Plate	<1

Active Air Sampling

- Exposure time- 10 min TBC- 100 CFU/m^3
- TFC- 10 CFU/ m^3

Table 3: Active Air Sampling

Area	TBC	TFC
Gowning Room	20 CFU/ m^3	<1
Media preparation room	14 CFU/ m^3	<1
Fabrication	10 CFU/ m^3	<1

DISCUSSION AND CONCLUSION

Environmental testing systems have tested the bio-burden of the aseptic or regulated environment, to understand the various issues relating to the aseptic treatment of bulk drugs (sterile), dose types, medical equipment, and to the establishment, preservation and regulation of the microbiological quality in the controlled environment.

Environmental monitoring is performed by two method.

1. Active Air Sampling

2. Passive Air Sampling

Forced air sampling is also known as successful air sampling. A fixed volume of air is sampled in this process or sampled indirectly on plates containing media. For successful air sampling, air samplers are often used. (Settle plate method) Passive air sampling. Directly exposed to environmental conditions in these media plates. The exposed plates are now incubated and the plates are counted directly.

Environmental testing has been carried out in order to verify the bio-burden of the aseptic region in order to avoid contamination of the goods being produced. In terms of GMPs, Good Laboratory Procedures, climate (aseptic cleanroom ISO class 5 or better), and employee practises, sterility testing requires high degree of control. In the practise of sterility testing, it is critical that meticulous techniques are used. Sterility checking, as well as routine quality control, is an important part of sterilisation validation. In general, false positive findings in the testing of drug products using a closed method are rare.

In order to prevent further contamination of the sample specimen, the microbes obtained from the equipment should be kept in pre-sterilized containers during a microbial sample. During the final analysis, a contaminated specimen could lead to biassed results. The importance of microbiology in the validation of cleaning has been noted by various regulatory bodies over the years. Before, during and after the cleaning process, a lot of focus is put on comprehensive microbial studies and study.

Why Microbiology is Necessary during Cleaning Validation

The entire validation process of cleaning mainly includes the collection, storage, research and examination of the various collected specimens, cleaning on different parts of the unit. They research and measure the microbial contents of these specimens to each other. The microbial content value of a contaminated sample would be different from that of an uncontaminated sample. The main steps taken when studying these specimens is microbiology and should be done by individuals with industrial expertise to ensure thorough research.

Active residues contain elements which exhibit various microbial and clinical features. To assess the therapeutic value of their constituent

ingredients, the detergents that are used during the cleaning process should be examined by microbiology. Microbiology and Documentation during Cleaning Validation It is important to clearly record the microbiological process followed during the cleaning process. This ensures smooth and swift checking by regulatory bodies and avoidance of confusion during the whole process.

Microbiological research will remain one of the most significant steps in the validation of cleaning since the findings obtained will be used in many ways: deciding the technique to be used during the cleaning process, identifying various elements such as detergents to be used and ensuring that the amount of cleaning performed is adequate before the next production is performed. Microbial analysis is a very critical validation tool for cleaning and should be conducted by professionals with appropriate experience. People tend to be quality-focused in a rapidly increasing economy, and cleaning validation is performed on the manufacturing equipment often plays a major role in the final product quality. The CFU / plate limits after exposure (4 hours) in the working area of the plate are given below:

I continued to evaluate various environmental grades for the isolation and detection of the total viable microbial count over the course of those 3 months. However, the findings suggest that the environment is sufficient and sufficient for the production and processing of drugs.

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CONFLICT OF INTEREST

None

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