PHARMA DEVILS

MICROBIOLOGY DEPARTMENT

STANDARD OPERATING PROCEDURE	
Department: Microbiology	**SOP No.:**
Title: Isolation and Identification of Microorganism	**Effective Date:**
Supersedes: Nil	**Review Date:**
Issue Date:	**Page No.:**

1.0 OBJECTIVE

To lay down a procedure for isolation and Identification of Microorganism.

2.0 RESPONSIBILTY

Microbiologist./ Q.C Executive

3.0 ACCOUNTABILITY

Quality Control Manager

4.0 PROCEDURE

4.1 To isolate the microorganism ,streak plates of SCDA and SDA .Incubate the plate of SCDA At 37°C for 24-48 hours and SDA at 25°C for 120hours.

4.2 IDENTIFICATION OF BACTERIAL COLONY

4.2.1 Inoculate the isolated colony in Nutrient Broth and incubate it for 24-48 Hours and Preserve it for further tests.

4.3 MORPHOLOGICAL TESTTS (GRAM'S STAINING)

4.3.1 Prepare thin smear, dry in air and fix by gentle heat.
4.3.2 Flood the prepared smear with Gram's Crystal Violet for 1 minute.
4.3.3 Wash with water and flood with Grams Iodine for 1 minute.
4.3.4 Wash with water and decolouriser until no further violet colour Comes off.
4.3.5 Wash with water and counterstain with 0.5% Safranin for about ½ minute.
4.3.6 Wash with water, dry and observe under oil immersion objective.

4.3.7 RESULTS INTERPRETATION

Gram +ve Cocci/Rods

Gram –ve Cocci/Rods

4.4 MOTILITY (HANGING DROP METHOD)

4.4.1 Wax the peripheri of coverslip
4.4.2 Put small drop of Nutrient Broth culture on the coverslip using wire Loop.
4.4.1 Put grooved slide on the coverslip and invent the grooved slide so that Coverslip is on the top.
4.4.2 Focus the edge of hanging drop at 10X objective of microscope and

STANDARD OPERATING PROCEDURE	
Department: Microbiology	**SOP No.:**
Title: Isolation and Identification of Microorganism	**Effective Date:**
Supersedes: Nil	**Review Date:**
Issue Date:	**Page No.:**

Finally observe at 40X objective.

4.4.3 RESULTS INTERPRETATION

Motile / Non Motile Rods

Motile / Non Motile Cocci

4.5 BIOCHEMICAL TESTS CATALASE TEST

4.5.1 The test demonstrates the presence of catalase, an enzyme that catalyses the release of O_2 from H_2O_2.
Reagent: 3% H_2O_2

4.5.2 METHOD:

4.5.2.1 Put a drop of H_2O_2 solution on a clean glass slide. Pickup a small amount of culture to be tested from the colony with sterile thin glass rod or sealed capillary tube .

4.5.3 RESULT INTERPRETATION:

4.5.3.1 The production of gas bubbles indicates a positive reaction. A false positive reaction may be obtained if an iron wire loop is used.

4.6 OXIDASE TEST

4.6.1 The test demonstrate the presence of Oxidase and is used for screening the species of Neissria , Alcalgens , Vibro , CampylobacterAnd Pseudomonas which give positive reaction and for excluding the Enterobacteriaceae, all species of which give negative reaction.
Reagent :1% Tetra methyl- Paraphenylene diamine-Dihydrochloride, stored in Amber bottle.

4.6.2 METHOD:

4.6.2.1 Take an oxidase disc in a Petriplate and moisten it with distilled water. Pick up the colony to be tested with clean sterile glass rod and smear on the oxidase disc. Alternatively touch the disc to the colony.

4.6.2.2 RESULTS INTERPRETATION:

A positive reaction is indicated by change in colour within 5-10 seconds as appearance of deep purple blue . A delayed positive reaction appears in 10-60 seconds, while a change in colour later than 60 seconds or no colour change at all is considered negative reaction.

STANDARD OPERATING PROCEDURE	
Department: Microbiology	**SOP No.:**
Title: Isolation and Identification of Microorganism	**Effective Date:**
Supersedes: Nil	**Review Date:**
Issue Date:	**Page No.:**

4.7 If Gram –ve /+ve rods are observed in Gram"s staining, perform Indole, M.R., V.P. & Citrate (IMViC) tests.

4.8 INDOLE TEST:

4.8.1 The test demonstrate the ability of certain bacteria to decompose tryptophan, an amino acid, to Indole which accumulates in the medium . Indole is then tested for by a colorimetric reaction with p-dimethyl - aminobenzaldehyde present in the Kovac's reagent.
Reagent: Kovac's reagent

4.8.2 METHOD:

4.8.2.1 To 5ml of 24 Hour old peptone culture add 0.5ml of Kovac's reagent and shake gently.

4.8.3 RESULT INTERPRETATION:

4.8.3.1 Development of red colour ring in the top layer indicate positive reaction.

4.9 METHYL RED TEST:

4.9.1 Test to distinguish between the organism which produce and maintain a high acidity (pH about 4.5) and those producing initially lower acidity which than reverts to neutrality. For example E. coli grown on glucose phosphate broth is able to produce and maintain a high concentration of hydrogen ions while Aerobactor produces initially a lower concentration of hydrogen ion and then causes reversion towards neutrality by the decomposition of the organic acid to carbohydrate and possibly by the formation of Ammonium compound from protein.
Reagent : Methyl red reagent
Methyl red 0.1gm.
Ethyl alcohol (95.0% -96.0%) –300ml.
Dissolve the dye in alcohol and then add sufficient distilled water to make 500 ml .

4.9.2 METHOD :

4.9.2.1 To 5 ml of 24 Hour old culture in Glucose phosphate broth(MR-VP Medium) add about 5 drops of Methyl red reagent.

4.9.3 RESULT INTERPRETATION:

4.9.3.1 Development of bright red colouration indicates positive reaction while yellow colour development indicates negative reaction.
Weakly positive reaction is indicated by development of reddish
orange coloration.

4.10 VOGES-PROSKAUER (V.P) TEST:

4.10.1 Test for the production of acetyl methyl carbinol (acetoin) or its reduction product 2,3 butylene glycol ($CH_3CH0HCHOHCH_3$). Some organisms (e.g. Acetobactor) produces acetoin ($CH_3CHOHCOCH_3$) or its reduction product 2,3 butylene gycol ($CH_3CHOHCHOHCH_3$) from breakdown of glucose. On addition of alkaline solution (strong potassium hydroxide) the acetoin or 2,3 butylene glycol ($CH_3CHOHCHOHCH_3$) in the presence of atmospheric oxygen is oxidised to di acetyl, which in turn reacts with a substance in the peptone, probably the Guanidine group of Arginine to produce pink colour.

4.10.2 METHOD
4.10.2.1 To 5 ml culture of MR-VP medium add 1 ml of 40% KOH and 3 ml of 5% solution of α -napthol in absolute ethanol.

4.10.3 RESULT INTERPRETATION:
4.10.3.1 Development of pink colour in 2 to 5 minutes, becoming crimson in
30 minutes Indicates positive reaction.

4.11 CITRATE UTILISATION TEST:

4.11.1 The test demonstrates the utilisation of Sodium Citrate as a sole source of carbon and Ammonium salt as sole source of nitrogen.

4.11.2 METHOD
4.11.2.1 Inoculate the slope of Simmon's Citrate Agar with a straight wire
from Nutrient Broth culture and incubate at 37°C for 24 to 48 hours.

4.11.3 RESULT INTERPRETATION:

4.11.3.1 Positive reaction is indicated by change in colour of the medium

from green to deep blue. The negative reaction is indicated by no change in the colour of medium.The results of all the four tests are interpreted together as IMViC. e.g. IMViC. (-+-+)

4.12 TEST FOR LACTOSE FERMENTATION (for Gram's -ve rods):

4.12.1 The test demonstrate the Lactose Fermenting (LF) /Non Lactose Fermenting (NLF) character of the organism.

4.12.2 METHOD:

4.12.2.1 From the Nutrient Broth culture of suspected colony streak a plate of Mac Conkey Agar and incubate at 37°C for 24 to 48 hours.

4.12.3 RESULT INTERPRETATION:

4.12.3.1 Lactose fermenting character of the organism is incicated by development of pink colonies while non lactose fermenting character is indicated by development of colourless colonies.

4.13 O/F TEST (for Gram positive Cocci):

4.13.1 The test demonstrates the utilisation of carbohydrates as oxidative and / or fermentative.

4.13.2 METHOD:

Duplicate tubes of Hugh and Leifson medium are inoculated by stabbing; 1 tube is promptly covered with a layer of sterile melted petroleum jelly (yellow soft petroleum to a depth of 5 to 10 mm and both are incubated at 37°C for 24 to 48 hours.

4.13.3 RESULT INTERPRETATION:

If acid is produced throughout the media in the covered (anaerobic) as well as open (aerobic) tubes, the breakdown is fermentative. If acid is produced only at the surface of medium where conditions are aerobic, the attack on the sugar is oxidative. The acid production is indicated by change in colour from blue to yellow.

4.14 LECITHINASE TEST

4.14.1 The test demonstrates the LECITHINASE activity of the organism. The test should be performed if B.cereus is suspected.

Medium: Nutrient Agar, Sterile 85 ml
Egg yolk suspension 15 ml

Melt the Agar, cool to 55°C and egg yolk suspension and pour the plates.

4.14.2 METHOD

4.14.2.1 Inoculate the Egg Yolk Agar plates and incubate at 37°C for 24-48 hours.

4.15.3 RESULT INTERPRETATION

4.15.3.1 Wide zone of opalescence indicates lecithinase activity of the bacteria

4.15.3.2 After analysing the results of Gram' staining, Motility, Catalase, Oxidase, LF/ NLF, IMViC, o/f & Lecithinase, if necessary other appropriate biochemicals or differential media should be used to confirm the identification of the suspected organism.

5.0 REASON FOR REVISION

Harmonization of format.

6.0 TRAINING:

Trainer	--	Head – Quality Control
Trainees	--	Quality Control Chemists & Assistants
Period	--	One day

7.0 DISTRIBUTION:

Certified Copy No. 1 : Head of Department – Quality Control
Certified Copy No. 2 : Microbiology Dept.
Certified Copy No. 3 : For Record file
Original Copy : Head – Quality Assurance.

8.0 ANNEXURES:

Annexure-I Format for Disinfectant and Detergent Testing

9.0 REFERENCES:

Inhouse

PHARMA DEVILS

MICROBIOLOGY DEPARTMENT

STANDARD OPERATING PROCEDURE	
Department: Microbiology	**SOP No.:**
Title: Isolation and Identification of Microorganism	**Effective Date:**
Supersedes: Nil	**Review Date:**
Issue Date:	**Page No.:**

ANNEXURE-I

REFRIGERATOR TEMPERATURE MONITORING RECORD

MAKE- BPL FROST FREE			**SET TEMPERATURE OF DEEP-FREEZER: -10.0°C LIMIT:±2%** **SET TEMPERATURE OF LOWER CHAMBER: 4.0°C**			
DATE	**TIME**	**TEMP. OBSERVED (OF DEEP-FREEZER)**	**TEMP. OBSERVED (OF LOWER CHAMBER)**	**OBSERVED BY**	**CHECKED BY**	**REMARKS**
/ /	HRS.	°C	°C			O.K/NOT O.K.