Department	Micro Laboratory		Document no	MICLAB – METHOD 028	
Title	Identification of bacterial isolates and contaminants				
Prepared by:		Date:		Supersedes:	
Checked by:		Date:		Date Issued:	
Approved by:		Date:		Review Date:	

1.0 SUMMARY OF CHANGES

Version #	Revision History
MICLAB – METHOD 028	New

2.0 PURPOSE

This procedure describes the necessary steps required in the identification of bacterial isolates and contaminants.

3.0 SCOPE

This procedure is used to profile and identify bacterial isolates/contaminants obtained from microbiological tests performed in the microbiology lab.

4.0 RESPONSIBILITY \ BUSINESS RULES

This procedure is applicable to all microbiology laboratory personnel involved in the identification of bacterial isolates/contaminants at Caringbah.

5.0 PROCEDURE

5.1 Materials Required.

5.1.1 Suitable medium (eg. TSA, NA, SDA)
5.1.2 Microscope and auxiliary equipment
5.1.3 Chemical reagents and test kits for biochemical profiles
5.1.4 Staining reagents
5.1.5 Micro Identification work sheet

5.2 Work Instruction

5.2.1 Streak the isolate/contaminant to be identified onto suitable non-selective plating media, e.g Tryptone Soya Agar (TSA). Label the plate with the relevant information. Incubate this plate at a suitable temperature, e.g. 30°C ± 1°C for 24 hours. Fill out the relevant details required on the Micro Identification Work Sheet.

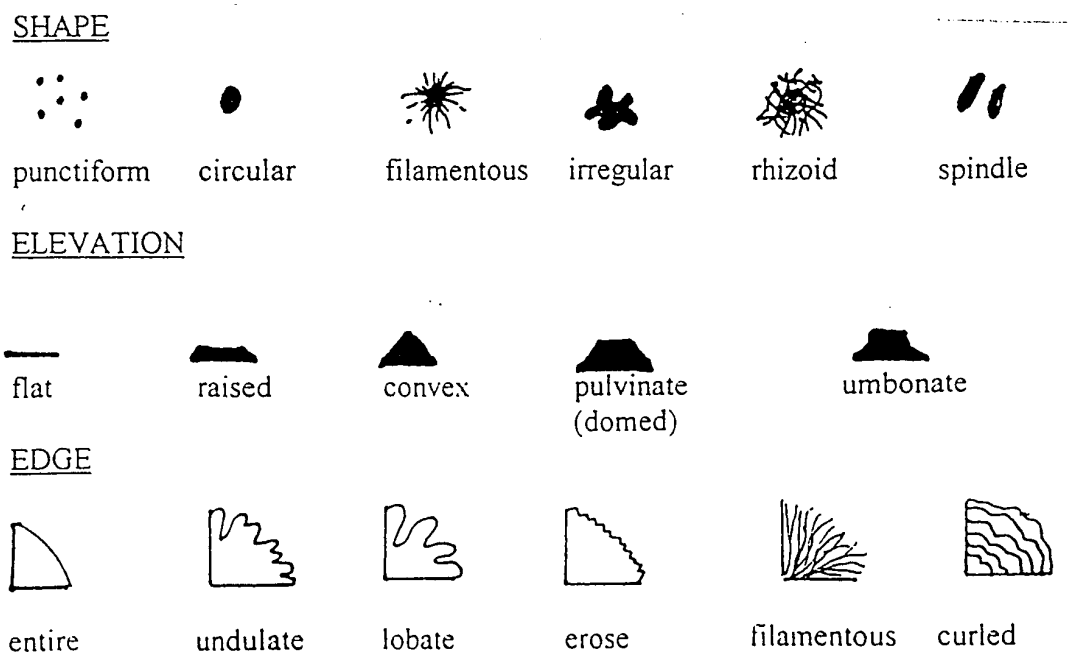
5.2.2 After the incubation time has elapsed, remove the plate from the incubator and ensure that the culture is pure.

5.2.3 With reference to Micro Identification Work Sheet, fill out the relevant details on this sheet. Determine colony morphology as per 5.3 and Gram stain reaction as per 5.4. Based upon the Gram stain reaction, follow the applicable steps to further identify the isolate.

NOTE: All identification work must be performed using gloves.

5.3 COLONY MORPHOLOGY CHARACTERISTICS

1. **Shape** — Circular, irregular, radiate, rhizoid, punctiform, spindle, filamentous.
2. **Size** — Record in μm or mm.
3. **Structure** — Amorphous, fine or coarsely granular.
4. **Surface** — Smooth, contoured, beaten-copper, rough, papillate. Dull or glistening.
5. **Elevation** — (as seen through vertical section) Flat, raised. Low convex, convex, umbonate, domed (pulvinate) with or without bevelled edge.
6. **Edge** — Entire, undulate, lobate, erose, effuse, filamentous.
7. **Colour/ Opacity** — Transparent, translucent, opaque.
9. **Consistency** — Butyrous, viscid, friable, membranous.
10. **Odour** — Present or absent, Resembling?
11. **Changes in Medium** — Colour change, digested, crystals formed, haemolysis on blood agar.
12. See Glossary of Terms for Colony Morphology Characteristics (Section 6.0).



5.4 GRAM STAIN TECHNIQUE

Using: Oxoid Gram Crystal Violet
Oxoid Gram Iodine
Oxoid 95% Ethanol/ Acetone (1:1)
Oxoid Gram Safranin

Perform a gram stain according to the following procedure.

5.4.1 Flood the fixed smear with Crystal Violet and stain for 1 minute.
5.4.2 Remove crystal violet by gently washing with cold tap water.
5.4.3 Flood the slide with iodine and retain for 1 minute.
5.4.4 Remove iodine by gently washing with tap water.
5.4.5 Decolourise by flooding the slide with ethanol / acetone (1:1) for 3 seconds.
5.4.6 Wash with cold tap water.
5.4.7 Flood the slide with safranin for 1 minute.
5.4.8 Wash the slide with cold tap water.
5.4.9 Blot or air dry the slide and view under the microscope.
5.4.10 If gram negative rods are obtained refer to 5.5.
If gram positive rods are obtained refer to 5.6.
If gram negative cocci are obtained refer to 5.7.
If gram positive cocci are obtained refer to 5.8.
If yeast are obtained refer to 5.9.

5.5 GRAM NEGATIVE RODS

Using the wet mount technique, determine if the isolate is motile and record % of motility.

5.5.1 Carry out an **oxidase test** by following the steps listed below:

Method A (Note: This method is not performed on a routine basis)

5.5.1.1 Place a small piece of Whatman filter paper No. 1 into an empty sterile petri dish.
Handle only the edges of the paper as oils from the skin can interfere with the reaction.

5.5.1.2 Pick up a few colonies of the test organism with a sterile loop and smear onto the filter paper. At the same time test a control culture of *Pseudomonas aeruginosa.*

5.5.1.3 Into a 10mL McCartney of sterile water, place a few granules of N,N,N,N'-Tetramethyl p-phenylenediamine dihydrochloride ($C_{10}H_{16}N_2$. 2HCL) (oxidase reagent) and mix thoroughly.

5.5.1.4 Pour the oxidase solution into the petri dish to moisten the paper, do not over flood.

5.5.1.5 A purple colouration must be observed within 30 seconds for an oxidase positive reaction.
No colouration indicates an oxidase negative reaction.

Method B

5.5.1.6 Place an oxidase strip using forceps into an empty sterile petri dish. Handle only the edges of the paper as oils from the skin can interfere with the reaction.

5.5.1.7 Pick one isolated colony using a sterile loop and smear the colony onto the oxidase strip.

5.5.1.8 A purple colouration must be observed within 30 seconds for an oxidase positive reaction.
No colouration indicates and oxidase negative reaction.

5.5.2 For gram negative, oxidase positive organisms, set up an API 20 NE identification strip to identify the organism to species level, where possible.

5.5.3 In order to aid the identification carry out an **O/F test** using the following procedure:

5.5.3.1 Stab inoculate two tubes of O/F medium with 10% filter sterilised glucose with isolate to be confirmed.

5.5.3.2 Inoculate two tubes each of the controls - *Pseudomonas aeruginosa* and *E.coli*.

5.5.3.3 Overlay one of each tube with sterile paraffin.
5.5.3.4 Incubate at 30°±1C for up to 72hrs.

5.5.3.5 If both the tubes are yellow the reaction is fermentative. If the tube that is not overlayed is yellow but the tube that is overlayed is green then the reaction is oxidative.

NOTE: *Pseudomonads* species are non fermentative.

5.5.4 For gram negative, oxidase negative organisms set up an API 20E identification strip to further identify the isolate. Note: *Xanthomonas maltophilia* is oxidase negative. If this isolate is suspected an API 20 NE should also be inoculated. If Salmonella spp is obtained from the API 20E profile, perform poly O and poly H antisera test.

5.5.5 The identification obtained from API may include the following comments: #

- **LOW DISCRIMINATION**: The profile corresponds to several very similar taxa – some supplementary tests may need to be performed.

- **NOT RELIABLE**: The sum of % id is less than 80. The profile is situated in a zone of confusion. Check that the strain belongs to the bacterial group being examined and that it is pure.

- **DOUBTFUL PROFILE**: A very atypical profile where there may be several tests against a proposed taxon. Check results for atypical tests. If correct the result could be due to a very rare biotype not previously encountered on the strip. Use additional information to confirm identification such as morphology, specimen source, supplementary tests etc. The culture could also be mixed

- **UNACCEPTABLE PROFILE**: The profile does not correspond to any taxon in the database. Check that the strain is pure as more than 2 tests at 0% against for the identification could mean that the culture is mixed.

NOTE: Always remember that it is extremely important to consider the identification comment even before considering the possible identification choices proposed. Never accept (without have done supplementary tests) an identification choice with a comment of "Low discrimination", "Not reliable", "Doubtful profile" or "Unacceptable profile".

BioMerieux API web version 1.2.1

5.6 GRAM POSITIVE RODS

5.6.1 From the gram stain result and the wet mount preparation note the presence or absence of any spores, the shape and position within the vegetative cell.

5.6.2 Perform a **catalase test** (if required) by following the steps listed below:

5.6.2.1 Onto a dry clean slide, place one drop of a Hydrogen Peroxide (3%) solution.

5.6.2.2 Using a sterile loop, pick up a few colonies of the test organism and emulsify with the hydrogen peroxide. At the same time run a positive control such as *Staphylococcus* sp.

5.6.2.3 Observe for the formation of air bubbles.

5.6.2.4 The presence of air bubbles indicates a catalase positive reaction.
No air bubbles indicates a catalase negative reaction.

5.6.3 Confirm the presence of spores (if required) by performing a malachite green spore stain as below.

5.6.4 **Spore Stain Technique**:

Using spore stain solutions 10% Malachite green (solution A) and 0.25% safranin O (solution B) perform a spore stain according to the following procedure.

5.6.4.1 Heat fix the prepared smear by passing the slide through a flame 20 times.

5.6.4.2 Let the slide cool then flood with Solution A and let stand for 10 minutes.

5.6.4.3 Rinse with tap water.

5.6.4.4 Flood slide with Solution B and let stand for 15 seconds.

5.6.4.5 Lightly rinse with tap water, blot dry and examine.

5.6.4.6 Record spore location (terminal, sub terminal or central) and look for swelling or distension of the cell.

5.6.5 Based on colony morphology and results of 5.6.1 and 5.6.2, if *Bacillus* species is suspected, inoculate an API 50 CHB identification strip for further identification.

5.6.6 Other non-*Bacillus* isolates should be sent to an external laboratory for further identification. For gram positive rods which are able to be isolated anaerobically (eg Clostridium, Bacillus), consult further reference literature.

5.7 GRAM NEGATIVE COCCI

5.7.1 Such isolates are usually found in clinical specimens. Isolation of these species is unlikely in a clean pharmaceutical plant. If such isolates are found these should be sent to an external laboratory for identification.

5.8 GRAM POSITIVE COCCI

5.8.2 Note the cell arrangement from the gram stain ie. pairs, tetrads, grapes, clusters, chains.

5.8.2 Carry out a catalase test (refer to 5.6.2.1). Catalase negative isolates belong to the streptococci group and should be sent to an external laboratory for further identification if required.

5.8.3 Catalase positive isolates belong to the *Staphylococcus* and *Micrococcus* groups.

5.8.4 Isolates identified as *Staphylococcus* species should be further classified as *S. aureus* or other *Staphylococcus* species using the **staphylase latex slide agglutination te**st following the instructions on the enclosed leaflet.

5.8.5 Any gram positive, catalase positive cocci which is positive for the agglutination test should be further identified using the STAPH API identification kit.

5.9 YEAST AND FUNGI

5.9.1 To confirm suspect yeast colonies, prepare a wet mount for phase contrast microscopy. Observe the cell shape and configuration. Note yeast cells are large and often budding. If more specific identification is necessary the culture should be sent to an external laboratory for further identification.

5.9.2 To identify fungal isolates (if required) prepare a wet mount using Lactic Acid for phase contrast microscopy. Observe the fungal hyphae, spores and reproductive structures. The characteristics noted from the observation made under phase contrast microscopy should then be confirmed using reference literature. If more specific identification is required the culture should be sent to an external laboratory for further identification.

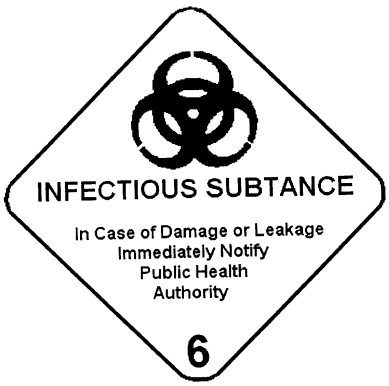
5.10 FURTHER IDENTIFICATIONS/TRANSPORTATION OF MICROBIOLOGICAL ISOLATES

If further identification from an external laboratory is required then the following transportation requirements must be adhered to.

5.10.1 Obtain and complete a declaration form (forms can be obtained from a post office).

5.10.2 Place the plate/slope in a sealed leak proof bag.

5.10.3 Pack sealed bag in an appropriate size package and attach an infectious substances (No. 6) label to the package.



5.10.4 Attach declaration form to the package in a self adhesive invoice envelope.

5.10.5 Send package through the internal mailing system.

5.11 Upon the completion of all identification tests, ensure that all the information gathered is recorded on the Micro Identification Work Sheet (SF150110). A copy of this Work Sheet should accompany the micro report form for this product/sample.

5.12 Inform the microbiologist of the identification of the isolate so that its significance to product quality and safety can be ascertained

6.0 DEFINITIONS / ACRONYMS

GLOSSARY OF TERMS FOR COLONY MORPHOLOGY CHARACTERISTICS

Amorphous Without visible differentiation into structure.

Auriculate With ear-like lobes.

Beaded	In culture-disjointed or semi-confluent colonies along line of inoculation. In stained smears - deeply staining granules in row.
Beaten Copper	With multiple small cratifiform depressions on the surface of growth.
Bevelled	Having a slanting edge.
Butyrous	Growth is of butter-like consistency.
Chains	Four or more bacterial cells attached end to end.
Contoured	An irregular smoothly undulating surface like that of a relief map.
Citron	Shaped like a lemon; ovoid with a small knob at the end.
Convex	Surface is rounded outwards.
Crateriform	A saucer shaped liquefaction of the medium.
Crenated	Refers to colony edge, intermediate between lobate and erose.
Cretaceous	Growth opaque and white, chalky.
Effuse	Growth thin and veil-like usually spreading. If referring to colony edge, flat spreading edge developing around central dense growth.
Endospores	Thick-walled spores formed within bacterial cells.
Entire	Colony with an even margin.
Erose	Boldly or irregularly toothed.
Filaments	Morphologically thread-like and usually unsegmented. If segmented, distinguished from chains by the absence of constrictions between the segments.
Filamentous	Growth composed of long, irregularly placed or woven threads.
Raised	Growth thick and comparatively flat with abrupt or terraced edges.
Reticulate	Like the veins of a leaf.
Rhizoid	Growth is of irregular branched or root-like structure.
Ring	Growth at the upper margin of a liquid culture, adhering to the glass.
Ringed	Having one or more circular depressions or elevations on the surface, sometimes giving a draughtsman-like appearance.
Rough	A general term for an irregular surface; resembling moroccan leather.
Rugose	Wrinkled.
Saccate	Liquefaction is in the form of an elongated sac, tubular or cylindrical.
Spreading	Growth extending much beyond (several millimetres or more) the line of inoculation.
Stratiform	Liquefying to the walls of the tube at the top and then proceeding downwards horizontally.
Translucent	Objects can be seen although the amount of light passing is appreciably reduced.

Truncate	Ends abrupt, square.
Turbid	Cloudy, may be uniform, flocculent or granular.
Umbonate	Having a button-like raised centre.
Undulate	Border is wavy with shallow sinuses.
Viscid	Growth follows the needle when touched, and on shaking broth cultures, the sediment rises with coherent swirl.

