# Aim

To describe the framework and individual tests for identification of microorganisms cultured in the diagnostic microbiology laboratory.

# Principle

Following isolation in pure culture on non-selective media and confirmation of microscopic characteristics by Gram stain, various biochemical and/or serological tests may be used to identify pathogens.

This SOP does not include details of identification of strict anaerobic species.

Other useful resources for background information and assistance with identification of difficult organisms include the following textbooks (available in the laboratory):

* Identification of Pathogenic Fungi (PHLS).
* Koneman's Colour Atlas and Textbook of Diagnostic Microbiology (Winn et al).
* Manual of Clinical Microbiology (Murray et al).
* Manual for the Identification of Medical Bacteria (Cowan and Steele).

# References

1. Health Protection Agency, UK SOP ID1: Introduction to the Preliminary Identification of Medically Important Bacteria (Issue 1.5; October 2011).
2. Cheesbrough M. District Laboratory Practice in Tropical Countries, Part 2. 2nd Edition Update (2006). Cambridge University Press.
3. Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA (ed.). Manual of Clinical Microbiology. 9th Edition (2007). ASM Press.
4. Commercial test kit inserts.
5. Standard Operating Procedures from LOMWRU, SMRU and AHC.

# Gram positive bacteria

## Gram positive cocci



### GPC algorithm 1



### GPC algorithm 2



## Gram positive bacilli

### Non-sporing bacilli

Non-sporing Gram positive bacilli may be significant pathogens in many sites and care should be taken to accurately identify them wherever possible: although they are also frequently skin contaminants. Discuss on the board.

* Perform a catalase test.
* Perform a bile aesculin test.
* Inoculate a TSI slope if the isolate is catalase negative.
* Set up a motility test.
* Observe any haemolysis on blood agar (aerobic and anaerobic) and note the cellular morphology.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Catalase** | **Haemolysis** | **Bile Aesculin** | **H2S production** | **Motility** | **Report** |
| + | - | - | - | - | *Corynebacterium* sp. |
| + | β | + | - | +\* | *Listeria* sp. |
| - | - | V | - | - | *Lactobacillus* sp. |
| - | α | - | + | - | *Erysipelothrix* sp. |
| - | β (at 48h) | - | - | - | *Arcanobacterium* sp. |

*\* Listeria* produces a typical “tumbling” motility at room temperature.

*\*\* Listeria*, *Erysipelothrix* and *Arcanobacterium* may have their identity confirmed by using an API Coryne strip.

### Screening for C. diphtheriae

Most corynebacteria, including the diphtheria bacilli, give rise to grey to black colonies on Hoyle’s tellurite medium and have a faint smell of garlic. All such isolates must be Gram stained to look for palely staining Gram positive bacilli (some staphylococci and enterococci also may grow on this medium).

* Perform a catalase test. Sub-culture on to a urea slope and incubate aerobically at 35-37°C:
* If urease positive at 4h, discard and report as negative.
* If urease negative at 4h, set up an API Coryne and sub-culture onto blood agar.

### Nocardia species

*Nocardia* speciesmay be recovered from cases of pulmonary disease or, rarely, brain abscess in patients who are immunocompromised or who are on aggressive steroid therapy. Other sources of these organisms include destructive subcutaneous infections (mycetomas) from which pigmented sulphur granules may be found in the pus taken from such lesions.

Filamentous, beaded organisms of pleomorphic morphology may be seen in smears of lesion material. Coccobacillary forms may also be observed. These organisms are partially acid-fast and this will raise the index of suspicion that the organism may be one of the nocardias.

Cultures may be made on Sabouraud agar (without chloramphenicol) incubated in air at 42°C as well as ordinary blood agar incubated aerobically at 37°C.

Cultures on blood agar at 48 hours will give rise to small white colonies that produce a musty, earthy smell. Colonies develop larger with increased incubation and give rise to heaped colonies and becoming darker in colour. An acid fast stain at this stage will confirm the partial acid-fast nature of the isolate. Growth on the Sabouraud agar will yield heaped colonies that develop a tan to orange colour.

Note: *Rhodococcus equii* may give rise to confusion. This organism is partially acid-fast, is coccoid in appearance and colonies may develop a pink pigment on prolonged isolation. Rhodococcus can be identified using the API Coryne kit.

### Bacillus species

*Bacillus anthracis*, a very rare isolate and the cause of anthrax, is the only member regarded as a pathogen in all circumstances and is a Hazard Group 3 organism. Any isolate from a skin lesion or a “black eschar” should be treated with caution and the work continued within a Class II biosafety cabinet.

Bacillus species are aerobic spore bearing Gram positive bacilli. Colonies on blood agar are usually large with a dry or crinkled surface. Some may produce moist or even mucoid colonies. The majority of isolates are oxidase positive.

Differentiation from the strains of clostridia which grow in air may be necessary. If this is required:

* Perform a catalase test.
* Subculture to blood agar aerobically and blood agar anaerobically (plus MTZ 5 disc), and Gram stain and look for spores.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Catalase** | **Spores (O2)** | **Spores (ANO2)** | **MTZ (5μg)** | **Report** |
| + | + | - | R | *Bacillus* sp\* |
| - | - | + | S | *Clostridium* sp\*\* |

\* grow better in air

*\*\** grow better anaerobically

Colonies of *B. anthracis* are irregular in shape, non-haemolytic and waxy in consistency. Gram stain reveals Gram positive bacilli with central spores giving a “bamboo” appearance. They are sensitive to penicillin. Gram staining of growth from around the penicillin disc gives rise to an alteration in morphology to a “string of pearls” appearance.

# Gram negative bacteria



## Gram negative cocci



## Gram negative bacilli / coccobacilli

### Fastidious organisms

#### Haemophilus spp.

*Haemophilus* species will grow more luxuriantly on chocolate agar than on blood agar and possess a typical “wet handkerchief” smell. On mixed cultures they may show “satellitism” around other bacterial colonies, particularly staphylococci.

Set up a test for factor requirements on nutrient (or Columbia / DNase) agar.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **XV** | **V** | **X** | **Haemolysis** | **Report** |
| + | - | - | - | *H. influenzae* |
| + | - | - | + | *H. haemolyticus* |
| + | + | - | - | *H. parainfluenzae* |
| + | - | + | - | *H. ducreyi* |

A test for β-lactamase is required for all haemophili (from around the ampicillin disc on the sensitivity plate).

If an isolate of *H. influenzae* is recovered from a child of <6 years of age check to see if it belongs to capsular type B using the respective antiserum. When a full biochemical identification of a haemophilus is required, or when factors have failed and the identification is imperative set up an API NH.

#### Other fastidious GNB/CB

Identification of other fastidious GNB/CB is often difficult: refer to appropriate references for advice.

### Non-fastidious organisms



Key biochemical reactions for GNB/CB are summarised below.

| **Organism** | **TSI** | | | **MIL** | | | **Citrate** | **Urea** | **Oxidase** | **Comment** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Butt/Slope** | **Gas** | **H2S** | **Indole** | **Motile** | **LDC** |
| ***Acinetobacter* spp.** | K/K or NC | - | - | - | - | - | + | - | - |  |
| ***Burkholderia pseudomallei*** | K/NC | - | - | - | + | - | NA | - | + | Wrinkled |
| ***Citrobacter diversus / koseri*** | A(K)/A | + | - | + | + | - | + | V | - |  |
| ***Citrobacter freundii*** | A(K)/A | V | V | V | + | - | V | V | - |  |
| ***Citrobacter* spp.** | A(K)/A | + | V | V | + | - | V | V | - |  |
| ***Edwardsiella tarda*** | K/A | + | + | + | + | + | - | - | - |  |
| ***Edwardsiella tarda* biogroup 1** | A/A | V | - | + | + | + | - | - | - |  |
| ***Enterobacter aerogenes*** | A/A | + | - | - | + | + | + | - | - |  |
| ***Enterobacter agglomerans* grp.** | K(A)/A | - | - | - | + | - | V | - | - |  |
| ***Enterobacter cloacae*** | A/A | + | - | - | + | - | + | V | - |  |
| ***Enterobacter* spp.** | A(K)/A | + | - | - | V | V | V | V | - |  |
| ***Escherichia coli*** | A/A | + | - | + | + | + | - | - | - |  |
| ***Escherichia coli* (inactive)** | A/A | - | - | V | - | V | - | - | - |  |
| ***Hafnia alvei*** | K/A | + | - | - | + | + | - | - | - |  |
| ***Hafnia alvei* biogroup 1** | K/A | - | - | - | - | + | - | - | - |  |
| ***Klebsiella oxytoca*** | A/A | + | - | + | - | + | + | + | - |  |
| ***Klebsiella ozaenae*** | K/A | V | - | - | - | V | V | - | - |  |
| ***Klebsiella pneumoniae*** | A/A | + | - | - | - | + | + | + | - | Mucoid |
| ***Klebsiella rhinoscleromatis*** | K/A | - | - | - | - | - | - | - | - |  |
| ***Klebsiella* spp.** | A/A | + | - | V | - | + | + | + | - |  |
| ***Morganella morganii*** | K/A | + | - | + | + | - | - | + | - |  |
| ***Morganella morganii* biogroup 1** | K/A | + | - | + | - | + | - | + | - |  |
| ***Proteus mirabilis*** | K/A | + | + | - | + | - | V | + | - | Swarming |
| ***Proteus penneri*** | A/A | V | V | - | V | - | - | + | - | Swarming |
| ***Proteus vulgaris*** | K(A)/A | V | + | + | + | - | V | + | - | Swarming |
| ***Providencia alcalifaciens*** | K/A | + | - | + | + | - | + | - | - |  |
| ***Providencia rettgeri*** | K/A | - | - | + | + | - | + | + | - |  |
| ***Providencia* spp.** | K(A)/A | V | - | + | V | - | V | V | - |  |
| ***Pseudomonas aeruginosa*** | K/K | - | - | - | + | - | NA | - | + | Blue-green |
| ***Pseudomonas* spp.** | K/K or NC | - | - | - | + | - | NA | - | + |  |
| ***Salmonella arizonae*** | K(A)/A | + | + | - | + | + | + | - | - |  |
| ***Salmonella choleraesuis*** | K/A | + | V | - | + | + | V | - | - |  |
| ***Salmonella* Paratyphi A** | K/A | + | - | - | + | - | - | - | - |  |
| ***Salmonella* Typhi** | K/A | - | + | - | + | + | - | - | - |  |
| ***Salmonella* spp.** | K/A | + | + | - | + | + | + | - | - |  |
| ***Serratia marcescens*** | K(A)/A | V | - | - | + | + | + | - | - |  |
| ***Serratia* spp.** | A(K)/A | V | - | V | + | V | + | - | - |  |
| ***Shigella* group A, B, C** | K/A | - | - | V | - | - | - | - | - |  |
| ***Shigella sonnei* (group D)** | K/A | - | - | - | - | - | - | - | - |  |
| ***Yersinia enterocolitica*** | A/A | - | - | V | - | - | - | V | - |  |

**Notes**

A = acid (yellow colour); K = alkaline (red colour); NC = No change

V = variable depending on strain; ND = not done

LDC = Lysine decarboxylase

Gas = bubble in bottom of tube

H2S = hydrogen sulphide (black pigment)

# Fungi

Fungi may be recovered from clinical material in three forms; yeasts, filamentous fungi (moulds) or dimorphic fungi (yeast at 37°C and a mould at 22°C).Significance is dependent on site of isolation and the patient’s clinical presentation.

## Yeasts

Perform a wet film or Gram stain to confirm that the colony is a yeast.

At this stage for most specimens “*Candida species*” or “yeasts” can be reported.

If further identification is required, perform a germ tube test.

*Cryptococcus* spp. must be considered when yeasts are cultured from immunocompromised (including HIV positive) patients. Perform a urea test to presumptively identify (*Cryptococcus* spp. are positive).

## Filamentous (mould) fungi

Care should be taken when examining cultures of filamentous or dimorphic fungi as many fungal isolates are Hazard Group 3 organisms (e.g. *Histoplasma capsulatum*, *Penicillium marneffii).*

Examine the culture within a Class II biosafety cabinet. Perform a wet mount in lactophenol cotton blue using a sellotape preparation to aid identification.

# Identification tests

## API Strips

See SOP MID-002 (Bacterial Identification Using bioMerieux API Kits).

## Aesculin Hydrolysis

### Principle / Intended use

The test is performed using bile aesculin agar and is used to screen for enterococci.

### Method

Pick a single colony for the test and spot inoculate a bile aesculin agar plate/slope with the colony.

Stab inoculate into the agar.

Incubate at 35-37°C in air and examine after 2-4 hours and again after overnight incubation.

### Interpretation

Enterococci give good growth together with blackening of the agar around the area of growth. Some bile tolerant organisms that do not hydrolyse aesculin may grow but will not blacken the agar.

Positive test: Growth and blackening.

Negative test: Growth but no Manner of Inoculation blackening, or no growth.

### Quality assurance

See media preparation SOP MED-001.

## β-Lactamase

### Principle / Intended use

Organisms showing resistance to β-lactam antibiotics may do so by the production of the enzyme β-lactamase. The presence of this enzyme is detected using the chromogenic cephalosporin nitrocefin.

### Method

Remove a tube of nitrocefin (Oxoid) from the fridge and place two drops onto some blotting paper.

Streak growth from around the Penicillin/Ampicillin disc across the blotting paper using a wire loop.

The development of a red colour indicates the presence of β-lactamase, this takes:

* Approximately 1 minute for *H. influenzae, N. gonorrhoeae* & *M. catarrhalis*
* Approximately 5 minutes for *E. faecalis*
* Approximately 30 minutes for anaerobic bacteria
* Approximately 60 minutes for *S. aureus*

Cefinase discs (BD) may also be used (streak a colony onto a disc moistened with sterile saline).

### Interpretation

Positive test: Red.

Negative test: Yellow/Colourless.

### Quality assurance

Positive control: *Staphylococcus aureus*.

Negative control: *Escherichia coli*.

## Bile Solubility

### Principle / Intended use

The bile solubility test is a confirmatory test used in the identification of *Streptococcus pneumoniae*.

### Method

Two tubes are required for bile solubility testing of each suspect strain of *S. pneumoniae*.

Take a loop of the suspect strain from fresh growth on a blood agar plate and prepare a bacterial cell suspension in 0.5 ml of sterile saline. The suspension of bacterial cells should be cloudy, similar to that of a 0.5 or 1.0 McFarland turbidity standard.

Divide the suspension into two equal amounts (*i.e.* 0.25 ml per tube). Add 0.25 ml of saline to one tube and 0.25 ml of 10% sodium deoxycholate (bile salts) to the other.

Shake the tubes gently and incubate them at 35°– 37°C for up to 2 hours.

Examine the tubes periodically for lysis of cells in the tube containing the bile salts. A clearing of the tube, or a loss in turbidity, is a positive result.

### Interpretation

Positive test: Clearing of turbidity (tube) or dissolution of colony (plate) - *bile soluble.*

Negative test: No clearing or colony dissolution - *bile insoluble.*

### Quality assurance

Positive control: *Streptococcus pneumoniae.*

Negative control: *Streptococcus mitis.*

## Burkholderia pseudomallei latex

See SOP MIC-013 (Isolation of *Burkholderia pseudomallei* from clinical specimens).

## Catalase

### Principle / Intended use

To detect the presence of the enzyme catalase. A useful test for differentiating streptococci (negative) from staphylococci or micrococci (both positive).

### Method

**Method 1**: Using a wire loop, smear the colony onto a clean glass slide. Place one drop of the reagent (3% hydrogen peroxide) onto a coverslip.

Invert the coverslip and place it over the smear so that the solution is contained underneath the coverslip. Examine for the immediate production of bubbles under the coverslip.

**Method 2:** Place one drop of reagent (3% hydrogen peroxide) onto a clean glass slide. Touch the colony under test with the corner of a coverslip and place this over the drop of reagent on the slide. Examine for the immediate production of bubbles under the coverslip.

A positive control (staphylococcus) should be used to test the activity of the reagent.

### Interpretation

Positive test: Immediate production of bubbles.

Negative test: No bubbles.

### Quality assurance

Positive control: *Staphylococcus aureus.*

Negative control: *Enterococcus faecalis.*

## Citrate

### Principle / Intended use

Detection of citrate utilisation for the differentiation of Enterobacteriaceae

### Method

Pick a single colony for the test and stab inoculate a citrate agar slope with the colony.

Incubate the slope at 35-37°C in air for 18-24 hours.

### Interpretation

Positive test: Blue colour.

Negative test: No colour change (green).

### Quality assurance

See media preparation SOP MED-001.

## Coagulase

### Principle / Intended use

Detects the presence of coagulase. Coagulase cross-links the α and β chain of fibrinogen in plasma to form fibrin clots. It enables the bacteria to stick to each other i.e. ‘clump’. This test is useful for differentiating Staphylococcus aureus (positive) from other staphylococci (coagulase-negative staphylococci; CoNS). The slide method detects bound coagulase (heat stable, clumping factor) and the tube method detects free coagulase (heat labile). Both types are produced by S. aureus. This SOP describes the use of Remel Staphaurex Plus (detecting both protein A and clumping factor), and the tube method (using Remel rabbit plasma and a modified protocol). The Staphaurex kit is used for screening and the tube method for confirmation.

### Method: Staphaurex Plus

Place a drop of the test latex reagent on a clean glass slide / test card.

Pick a suspect colony and mix with the latex reagent using a loop.

Mix for a maximum of 20 seconds (slowly).

Repeat using the control latex reagent

### Interpretation

Positive test: Agglutination of latex particles with background clearing in the test latex only.

Negative test: No agglutination/clearing in either test or control latex.

### Quality assurance

Positive control: *Staphylococcus aureus.*

Negative control: *Staphylococcus epidermidis.*

### Method: Tube coagulase

Add 18 drops of peptone water (or TSB / saline) and two drops of plasma to a glass tube.

Emulsify five colonies into diluted plasma and incubate for four hours at 37°C.

Set up positive, negative and reagent controls:

* *Positive*– *Staphylococcus aureus.*
* *Negative*– Coagulase negative staphylococcus.
* *Reagent*– one tube containing the uninoculated plasma peptone mixture.

Examine the tubes for formation of a clot.

Leave all tubes at room temperature overnight and recheck.

### Interpretation

Positive test: Formation of clot.

Negative test: No clot.

### Quality assurance

Positive control: *Staphylococcus aureus.*

Negative control: *Staphylococcus epidermidis.*

## DNase

### Principle / Intended use

This test is a confirmatory test for Staphylococcus aureus and should be performed on all staphylococci irrespective of the Staphaurex result. The DNase test is also a useful test for confirming the identity of tributyrin negative strains of M. catarrhalis. The test is performed as above but only after the DNA plate has been incubated for 48 hours to ensure detection of weak enzyme producers in this species.

### Method

Spot inoculate the colony under test onto a DNA plate and stab the growth in a criss-cross fashion. Include a known culture of *Staphylococcus aureus* as a positive control.

Incubate the plate overnight at 37°C in air.

The following morning flood the plate with 1% toluidine blue and read the result after 10 minutes.

### Interpretation

Positive test: A pink halo is seen around the growth.

Negative test: The medium around the colony remains blue.

### Quality assurance

See media preparation SOP MED-001.

## Factors (X+V)

### Principle / Intended use

This test is used to differentiate members of the Haemophilus genus by assessing growth around paper discs containing X (haemin) and V (NAD) factors in combination or separately.

### Method

Make a heavy suspension of the organism under test.

Dip a sterile swab into this suspension and swab the whole surface of a nutrient, Columbia, or DNase agar plate.

Place one of each of the factor discs (XV, X, and V) onto the surface of the plate and ensure that they are as far apart as possible.

Incubate the plate at 37°C in the CO2 incubator overnight and examine the next morning.

Only pure cultures must be read since any contaminants may serve as extraneous sources of V factor and give rise to false X factor results (i.e. mimics the XV result).

### Interpretation

For a positive result growth must occur around the disc completely. Growth around only half of the disc is likely to have been caused by discs being placed too close to each other and subsequent cross diffusion of factors.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **ID** | **XV** | **V** | **X** | **Haemolysis** |
| *H. influenzae* | + | - | - | - |
| *H. haemolyticus* | + | - | - | + |
| *H. parainfluenzae* | + | + | - | - |
| *H. ducreyi* | + | - | + | - |

### Quality assurance

Positive control (XV only): *Haemophilus influenzae.*

Positive control (XV and V): *Haemophilus parainfluenzae.*

## Germ Tube

### Principle / Intended use

*Candida albicans* can be distinguished from other Candida species by its ability to produce germ tubes.

### Method

Place approximately 0.5 ml of Remel Germ tube reagent (or horse serum) in a small plastic test tube.

Emulsify a small portion of yeast colony to be tested in the serum. Place cap on top of tube.

Incubate at 37°C, in the water bath for 2-4 hours.

Remove a drop of serum onto a slide, place a coverslip on top, and examine microscopically for germ tube production.

### Interpretation

Positive test: Germ tubes appear as cylindrical filaments originating from the blastospore, without any constriction at the point of origin and without obvious swelling along the length of the filament.

Negative test: No such structures seen.

Other species of Candida which grow germ tubes are *Candida dublinensis* and *Candida tropicalis* (*C. tropicalis* germ tubes are constricted at the point of origin from the yeast in contrast to *Candida albicans* which is not).

### Quality assurance

Positive control: *Candida albicans.*

Negative control: *Candida glabrata.*

## Motility

### Principle / Intended use

The motility test is used to aid identification of certain bacteria. Both negative and positive tests can be useful particularly in the identification of non-fermenting Gram negative bacilli when coupled with the OF test or when characteristic motility of any organism is examined (e.g. "tumbling" motility of *Listeria* spp.).

### Method

Pick a suspect colony into a tube of peptone water / TSB and leave standing at room temperature for four hours and examine using the method below.

If negative keep the culture at room temperature overnight and examine again the following morning.

Place one drop of suspension using a sterile pastette onto a coverslip and invert this onto a slide containing a ring of plasticine / blu-tak.

Examine as a wet preparation using the x40 objective and focussing on the edge of the drop.

### Interpretation

Motile: Active movement from one side of the field to another.

Non-Motile: No active movements as above including the "shimmering" appearance due to Brownian movement.

### Quality assurance

Not applicable.

## Motility-Indole-Lysine

### Principle / Intended use

MIL (motility-indole-lysine) medium is used for differentiating *Enterobacteriaceae* based on motility, lysine decarboxylation, lysine deamination and indole production.

### Method

Pick a single colony for the test and stab inoculate a MIL agar tube with the colony.

Incubate the tube at 35-37°C in air for 18-24 hours.

Add 2-3 drops of Kovac’s reagent (for indole production).

### Interpretation

Motility: Positive if the organism grows throughout the medium (diffuse growth, not a single line of growth).

Indole: Positive if pink colour forms after addition of Kovac’s reagent.

Lysine decarboxylase: Positive if the tube remains purple; negative if the agar becomes yellow.

### Quality assurance

See media preparation SOP MED-001.

## Oxidase

### Principle / Intended use

The oxidase test is a test used to measure the presence of cytochrome C3 (linked to cytochrome oxidase) in the bacterial cells respiration chain. It is a rapid test for helping to identify Pseudomonas, Neisseria, Vibrios, Aeromonads and other less commonly found bacteria.

### Method

Either touch the colony under test with an oxidase test paper or streak a colony picked with the corner of a coverslip over an oxidase test paper.

Alternatively touch a colony with a sterile swab moistened with oxidase reagent.

### Interpretation

Positive test: Purple colour.

Negative test: Colourless.

### Quality assurance

Positive control: *Pseudomonas aeruginosa.*

Negative control: *Escherichia coli.*

## Phadebact GC

### Principle / Intended use

A co-agglutination test for identification of *Neisseria gonorrhoeae* (to be used in parallel with bioMerieux API NH). The WI and WII/III Gonococcal Reagents are composed of two pools of murine monoclonal antibodies, reacting with a gonococcal specific membrane protein called protein I. Gonococci harbouring protein IA are classified to serogroup WI, whereas gonococci containing protein IB are referred to serogroup WII/III. The monoclonal antibodies of WI and WII/III Gonococcal Reagents are coupled to the protein A of non-viable staphylococci. When a sample containing gonococci is mixed with the WI and WII/III Gonococcal Reagent, the specific protein I antigens of the cell bind to the corresponding specific monoclonal antibodies. In this way a co-agglutination lattice is formed, which is visible to the naked eye.

### Method

Inoculate appropriate media (e.g. chocolate agar, GC selective agar) and incubate at 35-37°C in a humid atmosphere containing 5-10% CO2 for 16-24 hours.

Test only colonies presumptively identified as *N. gonorrhoeae* by biochemical tests (API NH).

Remove colonies from the primary plate to make a light suspension (0.5 McFarland standard) in 0.5ml of sterile saline.

Heat the liquid in a heating block / waterbath at 100°C for at least 5 minutes in a 1.5ml Eppendorf tube.

Cool the liquid to room temperature.

Pipette one drop of WI and one drop of WII/III Gonococcal Reagent onto separate circles on the supplied test card.

Add one drop of boiled colony suspension to each of the circles

Using separate pipette tips or a loop, mix each together thoroughly, and then rock the card gently for one minute observing for agglutination.

### Interpretation

Positive test: Visible agglutination with either WI or WII/III.

Negative test: No agglutination.

### Quality assurance

Positive controls: *Neisseria gonorrheaea* ATCC 19424 (serogroup WI) and ATCC 49498 (serogroup WII/III).

Negative control: By simultaneous use of both reagents in testing an unknown sample there is a built-in negative control since mixed infections are very rare.

## PGUA/Indole

### Principle / Intended use

The combined PGUA/Indole test is a rapid and cheap identification test for *E. coli. E. coli* is both PGUA and indole positive, which distinguishes it from all other coliforms.

### Method

Prepare a bacterial suspension (at least McFarland 4) from a pure overnight culture in 0.25 ml saline.

Add one PGUA/Indole tablet, close the tube and incubate aerobically at 35-37 °C overnight (although the test can be read after four hours if necessary).

First read the PGUA result, then add 3 drops of Kovacs' reagent, shake and read colour of the surface layer after 3 min (indole result).

### Interpretation

Positive test: PGUA – Yellow; Indole – Red.

Negative test: PGUA – Colourless; Indole – Yellow.

### Quality assurance

Positive control: *Escherichia coli*.

Negative control: *Enterobacter cloacae*.

## Pyrrolidonyl aminopeptidase (PYR)

### Principle / Intended use

The Pyrrolidonyl aminopeptidase (PYR) test is used to differentiate enterococci (positive) from streptococci (negative). The test can also be used as a screen for suspected *Staphylococcus lugdunensis*.

Enterococci produce pyrrolidonyl arylamidase, which hydrolyses the substrate L-pyrrolidonyl β-naphthylamide to produce β-naphthylamine. When the PYR reagent (4-dimethylaminocinnamaldehyde) is added to β-naphthylamine a pink / red colour change occurs.

### Method

Confirm that suspect colonies are catalase negative, Gram positive cocci.

Test colonies should be from pure 18-24 hour culture (slow growing isolates may be tested using 48 hour culture).

Prepare a heavy suspension (at least 4 McFarland) of the test organism in a tube containing 0.25 ml of sterile saline.

Add the PYR disc to the tube using forceps.

Seal the tube and incubate at 35-37 °C for 4 hours.

After incubation, add 3 drops of PYR reagent and observe for colour development within 5 minutes.

### Interpretation

Positive test: Pink, Red-Orange, Red colour change.

Negative test: Yellow, Yellow-Orange colour change.

### Quality assurance

Positive control: Enterococcus faecalis

Negative control: Streptococcus pneumoniae

## Slide Agglutination / Serotyping

*Salmonella* spp. and *Shigella* spp.: see SOP MIC-007 (Culture of faeces to identify enteric pathogens).

*Streptococcus pneumoniae*: see SOP MID-005 (Serotyping of *Streptococcus pneumoniae* isolates).

## Streptococcal Grouping

### Principle / Intended use

Identification of the Lancefield group of a beta-haemolytic streptococcal isolate using the Oxoid streptococcal grouping kit.

### Method

Add 0.4ml of reconstituted extraction enzyme solution to a clean tube.

Emulsify three or four colonies under test into the enzyme solution. Incubate at 37°C for 15 minutes to allow for digestion.

Place one drop of all six latex suspensions (A, B, C, D, F and G) onto the reaction card using the droppers provided.

Add one drop of the extract after the 15 minute digestion period to each well and mix gently.

Rock the slide back and forth for up to two minutes to look for agglutination of the latex particles.

Only one group latex should agglutinate.

Agglutination in two lanes may indicate too strong an extract. Repeat the test using a 1:5 dilution of the extract.

If the cross reactions persists or more than two suspensions agglutinate the test is invalid and should be repeated on a pure subculture. Some Group D strains also possess a Group G antigen and may give "cross reactions" with D and G antigens. These strains are rare but are true faecal streptococci and will give a positive aesculin reaction.

### Interpretation

Agglutination of a single latex reagent indicates its Lancefield group, e.g. Group B Streptococcus.

Group D positives should be confirmed by bile aesculin and reported as *Enterococcus* spp.

### Quality assurance

Use control latex as specific in the kit.

## Tributyrin

### Principle / Intended use

The tributyrin test is used as an aid for the identification of *Moraxella catarrhalis*.

### Method

Emulsify the organism under test into 0.25ml saline to give a suspension equivalent to a McFarland standard No.5.

Add a tributyrin tablet and incubate at 37°C for four hours.

### Interpretation

Positive test: Yellow colour produced.

Negative test: Red colour remains.

### Quality assurance

Positive control: Pseudomonas aeruginosa.

Negative control: Escherichia coli.

## Triple Sugar Iron

### Principle / Intended use

Triple Sugar Iron (TSI) test determines the ability of an organism to ferment sugars and to produce hydrogen sulphide (H2S) in anaerobic and aerobic conditions. A TSI slope contains three sugars (lactose, sucrose and glucose), phenol red (a pH indicator), sodium thiosulphate and an iron source (ferrous sulphate). If an organism is able to ferment any of the sugars, acid will be produced which will change the pH of the media and result in a colour change from red to yellow. The location of this colour change indicates if the organism is able to ferment the sugar under aerobic (slope) or anaerobic (butt) conditions. In addition, if the organism produces H2S (by reduction of sodium thiosulphate) this will react with the ferrous sulphate in the agar to form a black insoluble precipitate (ferrous sulphide).

### Method

Pick a single colony for the test and stab inoculate the butt of the TSI slope and on withdrawal “squiggle” the loop up the slope.

Loosely cap the slope

Incubate the slope at 35-37°C in air for 18-24 hours.

### Interpretation

|  |  |  |  |
| --- | --- | --- | --- |
| **Slant** | **Butt** | **Blackening** | **Result** |
| Red | Yellow | None | Ferments glucose |
| Yellow | Yellow | None | Ferments glucose and lactose and / or sucrose |
| Red / No change | Red / No change | None | No fermentation |
| Red | Yellow | Yes | Ferments glucose  H2S produced |
| Yellow | Yellow | Yes | Ferments glucose and lactose and / or sucrose  H2S produced |
| Red / No change | Red / No change | Yes | No fermentation  H2S produced |

Note:Blackening of the whole butt indicates large amounts of H2S. Blackening of the stab inoculation line indicates small amounts of H2S.

### Quality assurance

See media preparation SOP MED-001.

## Urea

### Principle / Intended use

Detection of urease production for the differentiation of Enterobacteriaceae. It can also assist in the presumptive identification of *Cryptococcus neoformans.*

### Method

Pick a single colony for the test and stab inoculate a urea agar slope with the colony.

Incubate the slope at 35-37°C in air for 18-24 hours.

### Interpretation

Positive test: Pink colour.

Negative test: No colour change.

### Quality assurance

See media preparation SOP MED-001.

# Risk assessment

|  |  |
| --- | --- |
| **COSHH risk assessment - University of Oxford COSHH Assessment Form** | |
| **Description of procedure**  Identification of cultured organisms using biochemical and serological tests | **Substances used**  Variable, depending on organism cultured (may include Gram / Ziehl-Neelsen stain reagents; 3% hydrogen peroxide (catalase test); Lactophenol blue; N,N,N',N'-tetramethyl-1,4-phenylenediamine (oxidase test); sodium deoxycholate (bile solubility test); bioMerieux API reagents) |
| **Quantities of chemicals used**  Small | **Frequency of SOP use**  Daily |
| **Hazards identified**  1. Cultures of pathogenic bacteria  2. Chemical hazards from the various biochemical identification tests | **Could a less hazardous substance be used instead?**  No |
| **What measures have you taken to control risk?**  1. Training in good laboratory practices (GLP)  2. Appropriate PPE (lab coat, gloves, eye protection)  3. Handling of specimens or cultures is done in the Class II biosafety cabinet until the presence of HG3 organisms (e.g. *B.* pseudomallei) have been excluded  4. Biological and chemical spill kits available in the laboratory | |
| **Checks on control measures**  Observation and supervision by senior staff | |
| **Is health surveillance required?**  No | **Training requirements:**  GLP |
| **Emergency procedures**:  1. Report all incidents to Safety Adviser  2. In event of any solution coming in contact with the eyes, immediately flush with running water for at least 15 minutes  3. If contact with skin, remove contaminated clothing and wash skin thoroughly  4. If any substance inhaled, move to fresh air and seek medical attention if any respiratory symptoms.  5. If ingestion of any substance, do NOT induce vomiting. If conscious, drink copious amounts of water, and seek medical attention if any gastrointestinal symptoms | **Waste disposal procedures**:  1. Sharps discarded into appropriate rigid containers for incineration  2. Infectious waste discarded into autoclave bags or 1% Virkon solution prior to autoclaving and subsequent incineration  3. Chemical waste disposed of according to manufacturer’s instructions and as documented in the AHC-COMRU MSDS database |