Introduction to Phenotypic Bacterial Identification Techniques

Mitchell Brown

Laboratory Diagnosis of Infectious Diseases: From Basics to Molecular Methods Workshop

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Westmead Education & Conference Centre

The Royal College of Pathologists of Australasia has received Australian Government funding under the Specialist Training Program.
The Identification Laboratory

• Small specialist identification laboratory
• Isolates from high-throughput general lab
• Unusual isolates from external labs
• Unique position to observe identification process in its entirety and understand the importance of basic phenotypic micro
Overview

• Where to start with an identification
• Importance of preliminary testing
• Examples and commonly encountered problems throughout
• Where to next…..
Identification Process

- Something not quite right?
- Start with the basics - get them right
- Solving the puzzle of a difficult organism
- Be aware of the limitations of any method
  - Databases for automated ID systems
  - Sequence databases – quality
But First- Two Tempting Traps…

- Total automation of phenotypic testing
  - Closed systems – bug goes in / identification comes out
  - Fundamentals such as metabolism can be ignored in enzymatic systems
  - Try to use in conjunction with manual methods

- Complete reliance on molecular techniques
  - Useful techniques discussed in detail by later speakers
  - Confirmatory/complimentary role should match phenotype
Useful Resources

• Textbooks -
  Manual of Clinical Microbiology;
  Koneman’s Colour Atlas and Textbook of Diagnostic Microbiology;
  Bergey’s Manual of Systematic Microbiology
  
  *Many more…..*

• Journal articles
• Databases
• Internet
The Time has Come to Proceed With a Formal Identification......

CIDM
Centre for Infectious Diseases & Microbiology
Public Health

RCPA
The Royal College of Pathologists of Australasia
Where & How Should We Start?
Primary Culture - Media

- Blood Agar
- $O_2$, $CO_2$ & AN
- MacConkey
- Chocolate
• Standardised preliminary identification sheet (will be available in lab, you may like to take one)

• Frequently return to basic phenotypic results, gram stains and growth conditions when deciding on final identifications.
Primary Culture – Record Growth

More on growth conditions further on when we discuss metabolism.

Growth Characteristics @ 35°C

<table>
<thead>
<tr>
<th>1. Growth in O₂</th>
<th>24hr</th>
<th>48hr</th>
<th>72hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBA</td>
<td>1+</td>
<td>2+</td>
<td></td>
</tr>
<tr>
<td>MAC</td>
<td>NG</td>
<td>NG</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2. Growth in CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBA</td>
</tr>
<tr>
<td>CHOC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3. Growth in ANO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBA</td>
</tr>
<tr>
<td>BHV</td>
</tr>
</tbody>
</table>

| 4. Other: |

Colonial Morphology: @ 24hrs ____________________________
@ 48hrs ____________________________
Colony Morphology

• Be descriptive:
• Flat, domed, spreading, entire, raised, shiny, umbonate, mat, rough, smooth, pitting, sticky, butyrous, waxy..... many more

Pasteurella multocida

From: ASM MicrobeLibrary.org © Robison, Moffitt, Thomson and Cohen
## Colony Morphology

<table>
<thead>
<tr>
<th>Form</th>
<th>Circular</th>
<th>Irregular</th>
<th>Filamentous</th>
<th>Rhizoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevation</td>
<td>Raised</td>
<td>Convex</td>
<td>Flat</td>
<td>Umbonate</td>
</tr>
<tr>
<td>Margin</td>
<td>Entire</td>
<td>Undulate</td>
<td>Filiform</td>
<td>Curled</td>
</tr>
</tbody>
</table>

From: [Google images](http://www.slic2.wsu.edu)
Colony Morphology – *Rothia dentocariosa*

‘Pinwheel’ shaped
Colony Morphology – *Eikenella corrodens*
Haemolysis
Haemolysis

- Destruction of red blood cells by $\alpha$- and $\beta$- haemolysins

*Streptococcus pyogenes*
β-Haemolysis Example –

*Clostridium perfringens*

Look for the characteristic double zone β – haemolysis. Hard to see here. Best on blood agar at 48hrs.
β-Haemolysis Example –

Arcanobacterium haemolyticum

Allows speciation;

A. haemolyticum – β
(may be weak)

A. pyogenes – β
(very strong)

A. bernardiae – α, β or Y
β-Haemolysis Example –

*Fusobacterium necrophorum*

Very strong β – Haemolysis.
Helps to speciate as *F. nucleatum* is non-haemolytic
α-Haemolysis Example –

*Actinomyces* spp.

- Remember to comment on haemolysis around individual colonies, not inocula
Haemolysis - Camp Test

- CAMP is an acronym for Christie, Atkins and Munch-Peterson, the Australians who first described this lytic phenomenon in 1944 for the presumptive identification of Group B Strep.
- Synergistic haemolysis - Diffusible extracellular protein (CAMP factor) and Staphylococcal β-Lysin
- The test has since been modified and can aid in the identification of Gram-positive rods including:
  - *Listeria monocytogenes*
  - *Corynebacterium* spp.
  - *Rhodococcus equi*
  - *Arcanobacterium* spp.
Camp test – Set-up
Camp Test – Example

*Corynebacterium coyleae*

*Corynebacterium coyleae*

*Staphylococcus aureus*
ATCC 25923

Zone of enhanced haemolysis
Pigments
Pigment – Use the Swab Method

Pigment may be hiding underneath
Some pigments may not be as obvious as this when observed on the plate.
Black pigment useful to separate from *Bacteroides* spp., Some strains take over 1 week.
Pigment – *Chromobacterium violaceum*

- Deep violet pigment
- Beware non-pigmented strains
Gram Stains
Where to Start - Gram Stain

• Don’t be afraid to do a gram stain!
• Be descriptive, recording
  – How does the organism stain?
  – Morphology
  – Cellular arrangement
  – Size, you may like to measure
  – Branching/rudimentary branching
  – Unusual features
How Does the Organism Stain?

• Is my organism Gram positive/ negative or Gram variable?

Gram positive – *Streptococcus* spp.
How Does the Organism Stain?

- Gram negative –

*Eikenella corrodens*
Gram variable? Can’t decide? –

- Don’t despair, there are several options available

![Bacillus cereus](image1.png) ![Moraxella spp.](image2.png)

*Bacillus cereus*  *Moraxella* spp.
Is the Organism Vancomycin Susceptible?

Resistant = Gram Negative

Sensitive = Gram Positive
### Vancomycin Exceptions

<table>
<thead>
<tr>
<th>Gram Positives</th>
<th>Gram Negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R</strong></td>
<td><strong>S</strong></td>
</tr>
<tr>
<td><em>Lactobacillus</em> spp. (some),</td>
<td><em>Flavimonas</em></td>
</tr>
<tr>
<td><em>Leuconostoc</em></td>
<td><em>Elizabethkingia</em></td>
</tr>
<tr>
<td><em>Weissella</em></td>
<td><em>Sphingomonas</em></td>
</tr>
<tr>
<td><em>Pediococcus</em></td>
<td><em>Empedobacter</em></td>
</tr>
<tr>
<td><em>VRE</em></td>
<td></td>
</tr>
<tr>
<td><em>Erysipelothrix</em></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium</em> spp. (some)</td>
<td>Also; occasional <em>Moraxella, Neisseria</em> and <em>Acinteobacter</em> spp.</td>
</tr>
</tbody>
</table>
Still Unsure? - Try the String Test

- Emulsify the organism in 3% KOH and observe for formation of a string
String test positive = Gram **Negative**

Thin cell wall allows KOH mediated lysis and formation of viscous strings of DNA)

String test negative = Gram **Positive**

Thicker cell walls are not lysed by the KOH

*Note: Sorry, again there are exceptions! Moraxella can be tricky*
How Does the Organism Stain?

- Another possibility. What if my organism fails to stain at all?

Consider *Mycoplasma hominis*
Morphology – Rod/ Coccobacilli /Cocci

• Look closely at the cellular morphology. Differences can be subtle
Sometimes it’s Easy….  

- rods  
  
- cocci (chain & cluster)
Sometimes it’s Not…

Unsure?
cocci or coccobacilli?
Try a Penicillin Challenge

- Gram stain from the edge of a zone to Penicillin
Coccobacilli elongate – *Acinetobacter, Moraxella*

True cocci form “puff balls” – *Neisseria*
Example – *Moraxella osloensis*

*M. osloensis* - Gram stain from edge of zone to penicillin - coccoid rods have become more elongated

*M. osloensis* very coccoid cells (from plate without penicillin)
Morphology - Cellular Arrangement

- Pairs, Chains, Clusters, Singular etc

- Check morphology in BHI/PYG broth
Morphology - Size

Subtle differences may be important – larger vs. smaller

Alloiococcus otitidis

Helcococcus kunzii
Morphology - Size

- fatter vs. thinner

“stocky” *Propionibacterium* spp.  more slender *Actinomyces* spp.
Morphology - Size

- longer vs. shorter

*Lactobacillus* spp.

*Listeria monocytogenes*
Morphology - Branching/Rudimentary

• Difference between the true branching of the aerobic actinomycetes (*Nocardia, Streptomyces* etc) and organisms with rudimentary branching, and irregular forms (*Actinomyces* and other irregular gram positive rods).
True Branching – *Nocardia* spp.

- True branching, at or close to right angles, sometimes tertiary branching.
True Branching – *Streptomyces* spp.

- Another example
Rudimentary Branching –

- *Actinomyces* spp.
Rudimentary Branching Precursors

- *Actinomyces* spp.
Unusual Features

• Several examples here but many more possibilities. These may help when referring to texts for identification.
Unusual features - Bifurcations

• or Bifid forms

not branching, more like a split end. Seen most commonly in Bifidobacterium spp.
Unusual features - Elbows

- Elbows – irregular feature common in *Rothia* & *Propionibacterium* spp., some other GPRs
Unusual features – Antibiotic Effect

Changes to morphology, usually due to interruption of cell division. Remember the Penicillin challenge? This is an *E. coli* in a B/C bottle.
Unusual Features – Fusiform Cells

- *Fusobacterium*, *Capnocytophaga* & *Leptotrichia* spp.
- Tapered ends
Unusual features – Bizarre Morphology

- Bizarre morphology- pleomorphism

*Streptobacillus moniliformis*

*Fusobacterium mortiferum*
Unusual features – Whip Handles

• Whip handles - *Corynebacterium matruchotii*
Unusual features – Spores

Gram or wet prep from the inocula, or from an older culture to see spores.

Spores must not be fully developed.
Unusual features – Spores

- *Bacillus* and *Clostridium* spp.
- Shape
- Position in sporangium
- Swelling of sporangium
Unusual features - B6 Dependence

- *or* Nutritionally Variant Streptococci (NVS)

Bulging rod forms and cocci, pleomorphic.

Consider the nutritionally variant streptococci
Unusual features - NVS

Addition of 250µL B6 to media can significantly improve morphology.

Also add to inocula for biochemical testing.
Unusual features -

Nutritional requirements

- Cysteine, Thymidine, Glutamine & many others
- Common in E. coli, especially in urine, easily overlooked
Unusual features - Why is my Organism Failing to thrive?

- Antibiotic effects
- Nutritional requirements B6, cysteine, thymidine
- SPS effect especially *Capnocytophaga* spp.
- Sub-optimal growth conditions – temperature or atmosphere

(*Campylobacter* spp., *Methyllobacterium* spp.)
Gram Example: *Actinomyces* spp.

Smallish, irregular GPR, rudimentary branching, nodules, some curved cells

Growth conditions will exclude *Rothia* spp.
Gram: *Anaerobiospirillum* spp.

- Spiral GNR
- Fatter and longer than most campy
- Strict ANO$_2$
Gram: *Actinomyces odontolyticus*

- Irregular GPR small/medium, rudimentary branching, nodules and curved cells
- Microcolonies in broth
- Red brown pigment with age
Gram: *Acinetobacter* spp.

- Plump medium
- GNCB singular and in pairs
- Pen challenge if unsure
- Ox neg a key
Gram: *Erysipelothrix rhusiopathiae*

- Small irregular gram variable rods, curvy.
- KIA reaction a key
Gram: *Bacillus cereus* group

- GPR, regular large and fat. Oval spores ST/C un-swollen sporangium
- (check WP)
Gram: *Clostridium clostridioforme*

- *Clostridium clostridioforme*
- Described as cats eyes
Growth Conditions and Metabolism
Growth Conditions

- Fundamental to placing your organism into a genus
- Suggests the organisms metabolism as fermentative or oxidative
- \( \text{O}_2 \rightarrow \text{CO}_2 \rightarrow \text{AN} (+ \text{a choc CO}_2 \text{ and a mac O}_2) \)
- Optimum temperature for growth
- Work with the organism where it is happiest, especially for Gram stain
Confirm Metabolism

- Use Kligler’s Iron Agar (KIA), Triple Sugar Iron Agar (TSI) or O/F glucose to confirm the metabolism of your organism is as the growth conditions might suggest.
Learn Your KIA Reactions

- Glucose oxidiser
- Glucose and lactose fermenter
- Glucose fermenter
- Fastidious glucose fermenter
Use O/F Glucose – Especially for Gram Positives

• They Don’t all Like KIA

Glucose oxidiser

Glucose fermenter
Example - Fastidious GNR – prefers CO$_2$
Example- Fastidious GNR – prefers CO$_2$

\textit{Haemophilus aphrophilus} – a fastidious glucose fermenter

KIA – A/NC $\rightarrow$ K/A
Example- Oxidative GNR – NG ANO₂

2+ Growth

3+ Growth

NG

O₂

CO₂

ANO₂
*Stenotrophomonas maltophilia*—a glucose oxidiser

O/F glucose – acidification only when exposed to air

KIA – K/NC
Propionibacterium acnes – Aerotolerant Anaerobe
Propionibacterium acnes – Aerotolerant Anaerobe

*Propionibacterium acnes* – a glucose fermenter
O/F glucose – acidification of both tubes
Corynebacterium pseudodiphtheriticum – Oxidative GPR

O₂: 2+ Growth
CO₂: 3+ Growth
ANO₂: No Growth
Corynebacterium pseudodiphtheriticum – Oxidative GPR

Corynebacterium pseudodiphtheriticum – a glucose oxidiser
O/F glucose – acidification only in aerobic tube, especially near top
A mixed genera. Includes both oxidisers and fermenters
Actinobaculum schaalii – Strong ANO₂ Preference

O₂  CO₂  ANO₂

+/- Growth  2+ Growth  3+ Growth
Actinobaculum schaalii – Strong ANO₂ Preference

Actinobaculum schaalii – a glucose fermenter
O/F glucose & KIA – unlikely to grow sufficiently
Not really necessary – all these Actino-like organisms are fermenters
Alloiococcus otitidis – Strictly Aerobic GPC
Alloiococcus otitidis – Strictly Aerobic GPC

*Alloiococcus otitidis* – asaccharolytic. Unusual for the streps/strep like bugs

KIA – unlikely to grow sufficiently
Bacillus circulans – struggling ANO$_2$ ?Oxidative
Bacillus circulans – struggling ANO$_2$? Oxidative

Bacillus circulans – a glucose oxidiser

O/F – acidification only in the aerobic tube. Weak ANO$_2$ growth suggests organism may be able to slowly or weakly ferment also. On extended incubation, O/F tubes may show this.
KIA reactions – other useful information

Look for Gas and \( \text{H}_2\text{S} \) also.
Interesting KIA reactions – *E. rhusiopathiae*

- Fastidious glucose fermenter
- The only fastidious GPR to produce H$_2$S (some *Bacillus* spp. do)
Interesting KIA reactions – *S. putrefaciens*

- *Shewanella* - the only oxidative GNR that produces H$_2$S in a KIA
- Oxidase, gram stain and KIA sufficient for ID
Additional Preliminary Testing

- Catalase test - positive
- Indole –positive (DMACA)
- Oxidase - positive
Oxidase

- Tests for the presence of the enzyme oxidase
- On a strip of filter paper, saturated with a few drops of oxidase reagent, smear a small amount of the test organism
- Don’t use selective media like MAC or XLD. False positives and negatives are common
Beware Oxidase

Negative Oxidisers

- *Acinetobacter* spp.
- Occasional *Burkholderia cepacia*
- *Pseudomonas luteola*
- *Pseudomonas oryzihabitans*
- *Burkholderia gladioli*
- *Stenotrophomonas maltophilia*
- There are others……..
Similarly, not all organisms which possess an oxidase are oxidative in metabolism, including:

- *Vibrio* (except *V. metschnikovii*)
- *Aeromonas*
- *Pasteurella* etc
Tests for the presence of the catalase enzyme, an enzyme which decomposes hydrogen peroxide into water and oxygen.

$$2\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} 2\text{H}_2\text{O} + \text{O}_2$$

Aerobes 3% $\text{H}_2\text{O}_2$  
anaerobes 15% $\text{H}_2\text{O}_2$  
Superoxyl ($N. \text{gonorrhoeae}$) 30% $\text{H}_2\text{O}_2$
False/

Pseudo Catalase

- Always perform catalase from chocolate, or a blood free media such as LD
- To detect weak catalase emulsify some organism in the catalase reagent on a slide and cover with a coverslip
- Enterococci and Lactobacilli can decompose peroxidase with a pseudocatalase
Indole

- Tests for the presence of Tryptophanase which is involved in the deamination of tryptophan to produce indole;

**Spot Indole** –

```
Tryptophan → Tryptophanase deamination → Indole
which turns p – Dimethylaminocinnamaldehyde or DMACA blue/green
```

**Kovac’s Indole** –

```
Tryptophan → Tryptophanase deamination → Indole
which turns p – Dimethylaminobenzaldehyde or DMAB pink/red
```
## Indole Example – *Escherichia coli*

| Spot indole | Kovacs Indole from peptone water | & from 1% Tryptophan broth |

![Image of spot indole test](image1.jpg)

![Image of Kovacs indole test](image2.jpg)

![Image of stronger reaction](image3.jpg)

*note: stronger reaction*
Morphology and Motility in Broth

- Check at 4 & 24hrs and always a direct wet prep
Example of Streptococcal morphology in broth @ 24hrs using 400x phase contrast microscopy
Morphology – Enhanced in Broth

Actinomyces spp. form microcolonies in broth
Motility – Setup

- Not too heavy – just like Gram stains!
- Allow the wet prep a little time to settle on the microscope stage so that you are in a single plane
- Have enough suspension so the cells aren’t squashed
- Brownian Motion

• Be careful not to overcall motility – ignore the ‘bouncing around’ of Brownian motion
• Look to see changes in direction, or cells moving in opposing directions
Motility

Unfortunately not always this obvious.

Video from:

Department of Microbiology & Immunology
University of Leicester, UK.
2001

©

CIDM
The Royal College of Pathologists of Australasia
Motility – Characteristic ‘Styles’

Look for characteristic types of motility;

- The tumbling motility of *Listeria monocytogenes*
- The ‘sluggish’ motility of *Bacillus megaterium* (usually not evident on direct wet prep)
- Darting motility of *Campylobacter* spp.
- Rare twitching motility of *Moraxella nonliquefaciens*
Motility – Temperature is Important

Remember flagella development is best at room temperature
Some *Yersinia* spp. including *Y. enterocolitica* are non-motile @ 37°C. There are also accompanying biochemical differences (VP neg at 37°C and pos at RT).
Gliding ‘Motility’

Capnocytophaga spp. has no flagella

Not really motility
More like a snail crawling across the plate
Presumptive identifications may be possible at this point, if only to genus level.
## Preliminary Identification

**Accession No:**      **External/Internal:** bench  
**Name:**  ISOLATE 3   
**Date Received:**  UNKNWN  

### Gram Stain: Tiny GNOC and cocci

<table>
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<td><strong>1. Growth in O₂</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Catalase: +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidase: -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAC No growth</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| **2. Growth in CO₂**          |       |       |       |
| BA +/- +/−                    |       |       |       |
| KIA: NC/NC or A/NC           |       |       |       |
| H₂S: -                        |       |       |       |
| GAS: -                       |       |       |       |
| * OF Glucose: oxidative / Fermentative |       |       |       |

### Motility: (BHI broth)

| RT  | 0 hr |       |       |
| RT  | 4 hr |       |       |
| RT  | 24 hr|       |       |

| Haemolysis: α, β, γ       |       |       |       |

### Pigment: Pale yellow (check pigment production @ 24 & 48hrs)

### Optimum Temperature for Growth: 37°C

### Colonial Morphology:

<table>
<thead>
<tr>
<th>@ 24hrs</th>
<th>0.1mm translucent to whitish</th>
</tr>
</thead>
<tbody>
<tr>
<td>@ 48hrs</td>
<td>1.0mm dry growth, white-grey, adherent</td>
</tr>
</tbody>
</table>

**Preliminary identification:** Presumptive HACEK organism - ? *Actinobacillus actinomycetemcomitans*
FINISHED? –Not Quite…

But we can now make an informed choice about how to proceed with identification – method selection etc.
Briefly - Identification to Species Level
Speciation

- Refer to textbooks/journal articles
- Use in-house tables/flow charts/databases
- Be patient - sometimes this takes time
- Are you confident of your preliminary ID
Remember Limitations of any Methods - Databases etc
Supplementary Methods for Speciation

- Many methods, kits and machines available
- Briefly explain a few that we use here at CIDM
- 16s, Maldi-tof, LCFAA
- Don’t forget good old-fashioned API, Vitek, Phoenix, Remel etc
16s rRNA gene sequencing

- Primers target conserved regions and amplify the intervening variable regions. Amplicons of ~1500bp are sequenced
- Very useful however no panacea!
- Inherent problem of non peer-reviewed sequences databases
- Will be discussed later in the day
Long Chain Fatty Acid Analysis

- Detect, identify and quantify cellular long chain fatty acids, using gas chromatography
- Generate dendrogram based on fatty acid profile allowing comparison with library entries
- Works well for certain genera
Maldi-Tof

- Matrix assisted laser desorption/ionisation – time of flight
- Protein profile based on time of flight of molecules ionised
- Being evaluated here at CIDM
- Fast
- Results preliminary but as with other methods seems promising for some genera and not so for others
Finally – A Practical Example….

Actinomyces neuii
ssp. neuii

vs.

Corynebacterium striatum
Culture

Similar appearance

Actinomyces neuii
ssp. neuii

Corynebacterium striatum
Actinomyces neuii ssp. neuii

Corynebacterium striatum

Gram
Growth

Different

Corynebacterium striatum

\( O_2 \) \( CO_2 \) \( ANO_2 \)

Actinomyces neuii ssp. neuii
In summary –

**Actinomyces vs. Corynebacterium**

- Colony appearance – can be similar
- Gram stain – is different
- Growth conditions – are different

Like chalk and cheese
Hard to confuse!
General tips ~ In summary

- Take the time to investigate when something seems wrong
- Get the basics right
- Remember the limitations of any method
- Refer to texts and other resources
- Source specialist media if you can’t manufacture your own
- Consult your local Reference Laboratory
Thankyou for listening
Any Questions?