Table of Contents

1. Introduction 3
2. Administration and Preparation of Isolates 3
2.1. Classification of Test Samples 3
2.2. Unique Sample Identifier 3
2.3. Preparing Isolates for ID 4
3. ID of Isolates 6
3.1. The Gram Stain 6
3.2. Technical Information 7
3.3. The Potassium Hydroxide (KOH) test 8
3.4. The Catalase Test 9
3.5. The Coagulase Test 10
3.6. Sporulation and The Spore Stain 11
4. Quality Control (QC) 12
4.1. QC and Traceability of Reagents/Kits 12
5. Documentation of Results 13
5.1. Results Recording and Further ID 13
6. Monitoring Trends 13
6.1. Trending of Microbial ID Results 13
7. Appendices 14
7.1. Colony Morphology 14
7.2. Cellular Shape and Arrangement 14
7.3. ID Flowchart: Gram Positive Cocci 16
7.4. ID Flowchart: Gram Positive Rods 17
7.5. ID Flowchart: Gram Negative Rods 18
7.6. ID Flowchart: Yeasts 19
7.7. Gram Positive Cocci 20
7.8. Gram Positive Rods – Sporing 22
7.9. Gram Positive Rods – Non-sporing 24
7.10. Gram Negative Organisms (Non-glucose fermenters) 25
7.11. Gram Negative Organisms (Glucose fermenters) 28
8. Summary of Changes 28

2.3.6. Flame a wire loop until red-hot by passing through the hottest part of a Bunsen burner flame OR take a sterile disposable loop. If using a wire loop, allow the loop to cool before proceeding (to cool the loop quickly touch the loop against the agar of the TSA plate to be used).

2.3.7. With the sterile loop, lightly touch the colony to be streaked OR take one loopful of a broth culture from the test sample and immediately streak the growth onto the TSA plate creating a primary inoculation well (Fig. 1a).

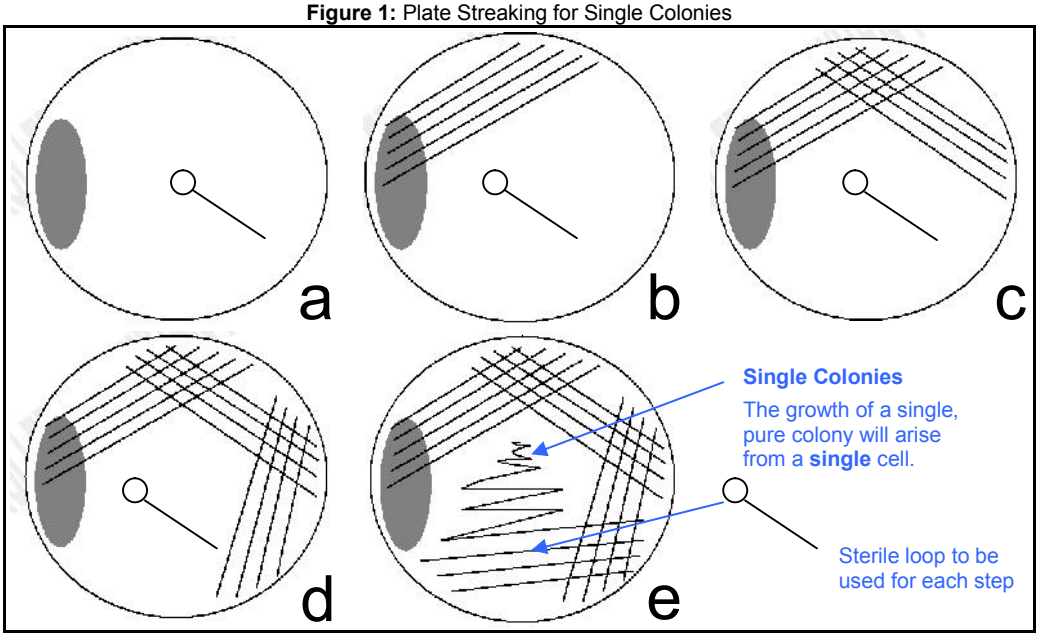
2.3.8. Flame the wire loop and allow to cool OR take a fresh sterile disposable loop and streak 4-7 parallel streak lines from the primary well (Fig. 1b).

2.3.9. Flame the wire loop and allow to cool OR take a fresh sterile disposable loop and streak 4 parallel streak lines from those streaked in step 1.4.6.3 (Fig. 1c).

2.3.10. Flame the wire loop and allow to cool OR take a fresh sterile disposable loop and streak 4 parallel streak lines from those streaked in step 1.4.6.4 (Fig. 1d).

2.3.11. Flame the wire loop and allow to cool OR take a fresh sterile disposable loop and streak 4 parallel streak lines from those streaked in step 1.4.6.5 (Fig. 1e). Extend the last streak line into the centre of the plate. *Note: A fresh sterile loop **must** be used for each new set of streak lines OR a wire loop must be flamed and cooled. Using a sterile loop facilitates the dilution of organisms with each set of streak lines enabling the growth of single colonies.*

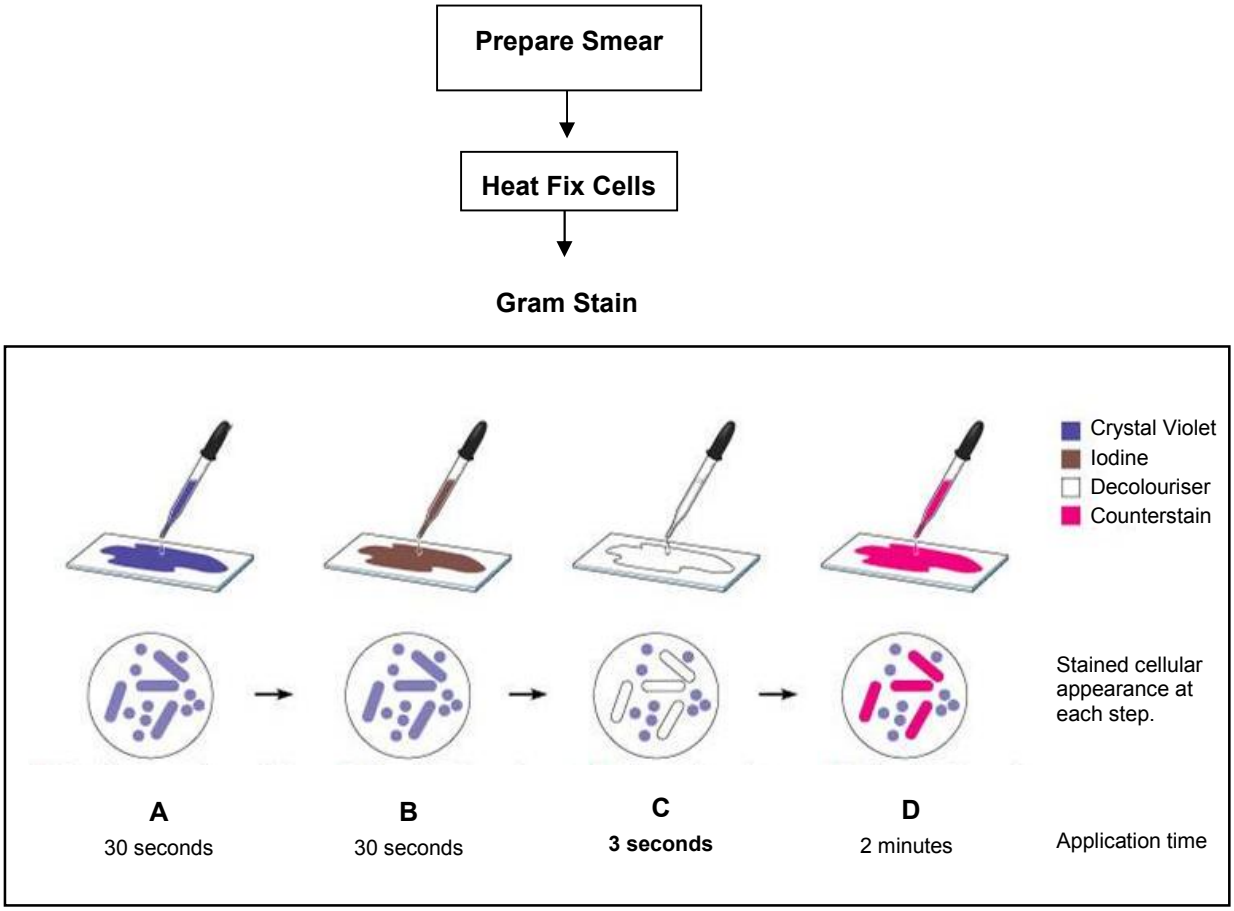
Figure 1: Plate Streaking for Single Colonies



2.3.11.1. Incubate all plates at 30-35ºC for 24 hours. Slow growing and stressed organisms may require longer incubation. Yeasts may require incubation for up to 7 days. Store the original test sample plate/broth labelled with the assigned USI at 2-8ºC until all ID work is complete. Some test samples will require a longer period of retention under these conditions (see Table 2). Ensure the integrity of all original test samples is preserved during storage.

Table 2: Test Sample Retention Periods

Figure 2: The Gram Stain



3.3. **The Potassium Hydroxide (KOH) test**

Known Gram-variable organisms and organisms which have lost some of their cell wall integrity (due to an ageing culture), appear Gram-negative on staining, resulting in possible misidentification.

The KOH test (also known as the "String Test") aids in the differentiation of Gram-positive bacteria from Gram-negative organisms.

In the presence of potassium hydroxide, Gram-negative cell walls are broken down, releasing viscid chromosomal material, which causes the bacterial suspension to become thick and stringy. Most Gram-positive organisms remain unaffected.

Note: The KOH test can only be performed using colonies grown on solid medium. ***The KOH test is to be performed on all non spore-forming rods and where the Gram reaction of any organism cannot be determined due to Gram variability or poor staining.*** *The KOH test is positive for 100% of all Gram-negative organisms but only 97% negative for all Gram-positive organisms. A negative result can only be obtained from a Gram-positive organism; a positive result can be obtained from all Gram-negative organisms and some Gram-positive organisms.*

3.3.1. Place one drop of 3% KOH solution on a clean microscope slide.

3.3.2. Using a sterile loop pick up 2-3 colonies and emulsify in the KOH to make a dense suspension. The suspension should appear cloudy if a sufficient number of colonies have been picked.

3.3.3. Mix the suspension continuously for up to 60 seconds, and then gently pull the loop away from the suspension. If the organism is Gram-negative the suspension will form a string which adheres to the loop and stretches from the slide (Fig. 3).

characteristics in terms of chemical and/or heat resistance. Sporulation is not always evident in fresh cultures grown on general-purpose media, as the conditions may not stimulate the formation of a spore.

It is of high importance that isolates collected from Filled Container Bioburdens (FCB) do not present any Gram Positive Sporing Rods. Any test samples presenting this type of growth will need a D-value performed. **Refer to MICLAB 075.**

To ensure that all GPRs are identified with accuracy, spore formation must be determined. **All Gram-positive rods collected from FCBs, which do not appear to be spore formers after culturing using TSA, are to be cultured using Sporulation Agar (SA). A spore stain may then be used for the demonstration of spores.**

Note: SA is a specialised medium used to stimulate bacterial spore generation by Bacillus spp. The medium contains peptones as a source of nitrogen, beef extract and yeast extract provide essential vitamins and growth factors for metabolism, while a low level of glucose provides a limiting source of carbon. The limited carbon source coupled with the addition of Manganese Sulphate stimulates sporulation.

3.6.1. Streak the isolate onto SA and incubate at 35-37ºC for 3-5 days.

3.6.2. Prepare a smear and heat fix the cells to the slide by passing the slide through the hottest part of the flame three times. Allow to cool.

3.6.3. Conduct a **Spore Stain** to observe spore formation. See **MICLAB 065**.

3.6.4. Examine the smear under Oil Immersion.

3.6.5. Interpretation

- **Positive** Spores stain green
- **Negative** Vegetative cells stain pink/red

Note: If sporulation is evident following culture on SA the organism is presumptively identified as a Bacillus spp.

If sporulation is not evident in the culture following 5 days incubation, the organism is presumptively identified as a Non-sporing Gram-positive rod.

3.6.6. Quality Control

Spore forming and non-spore forming reference cultures are to be used for quality control of the spore staining procedure and reagents.

QC organisms	
Positive	*Bacillus subtilis ATCC 6633*
Negative	*Escherichia coli ATCC 8739*

4. Quality Control (QC)

4.1. QC and Traceability of Reagents/Kits

4.1.1. For traceability purposes, the batch number and/or expiry date of all reagents used in characterisation tests are to be logged in the ID Reagents Logbook on a daily basis.

4.1.2. Reagents are to be challenged at each use (i.e. once daily) using the stated QC organisms (see Table 3). Staining reagents are to be challenged upon opening, using the stated QC organisms. They may then be verified for use.

4.1.3. The challenge organisms are to be **no more than 24 hours old**. Ensure a pure, fresh culture is always available for QC.

4.1.4. Record the QC results for all reagents in the ID Reagents Logbook.

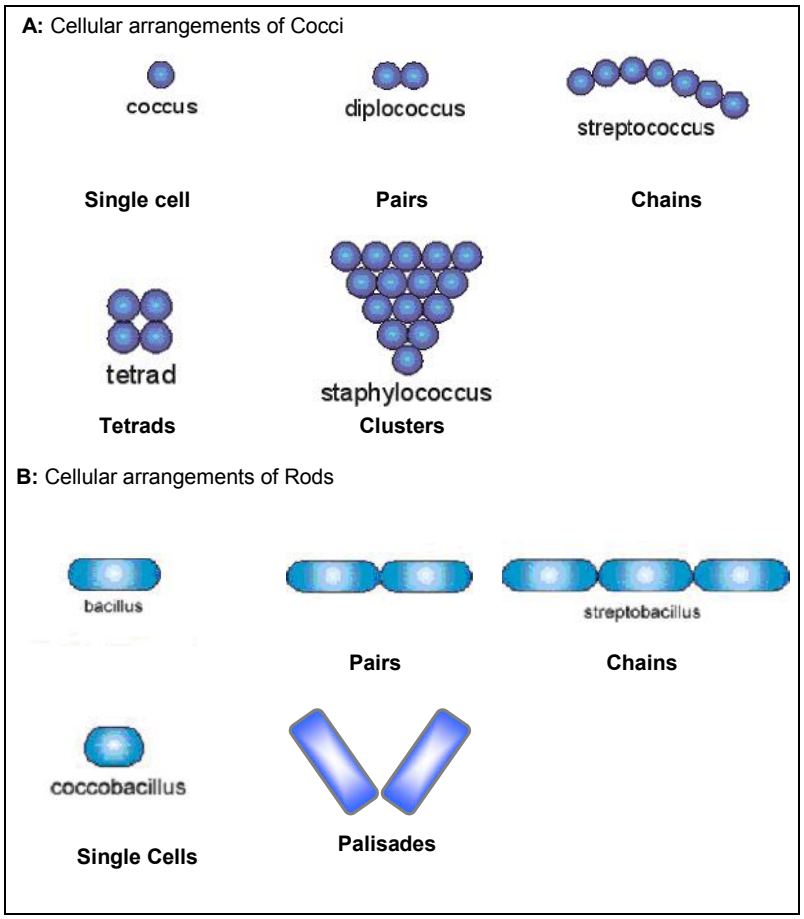
Coccus - The cocci are spherical or oval bacteria having one of several distinct arrangements based on their planes of division (Fig. 7A).

- Division in **one plane** produces either a **diplococcus** or **streptococcus** arrangement
- Division in **two planes** produces a **tetrad** arrangement
- Division in **random planes** produces a **staphylococcus** arrangement

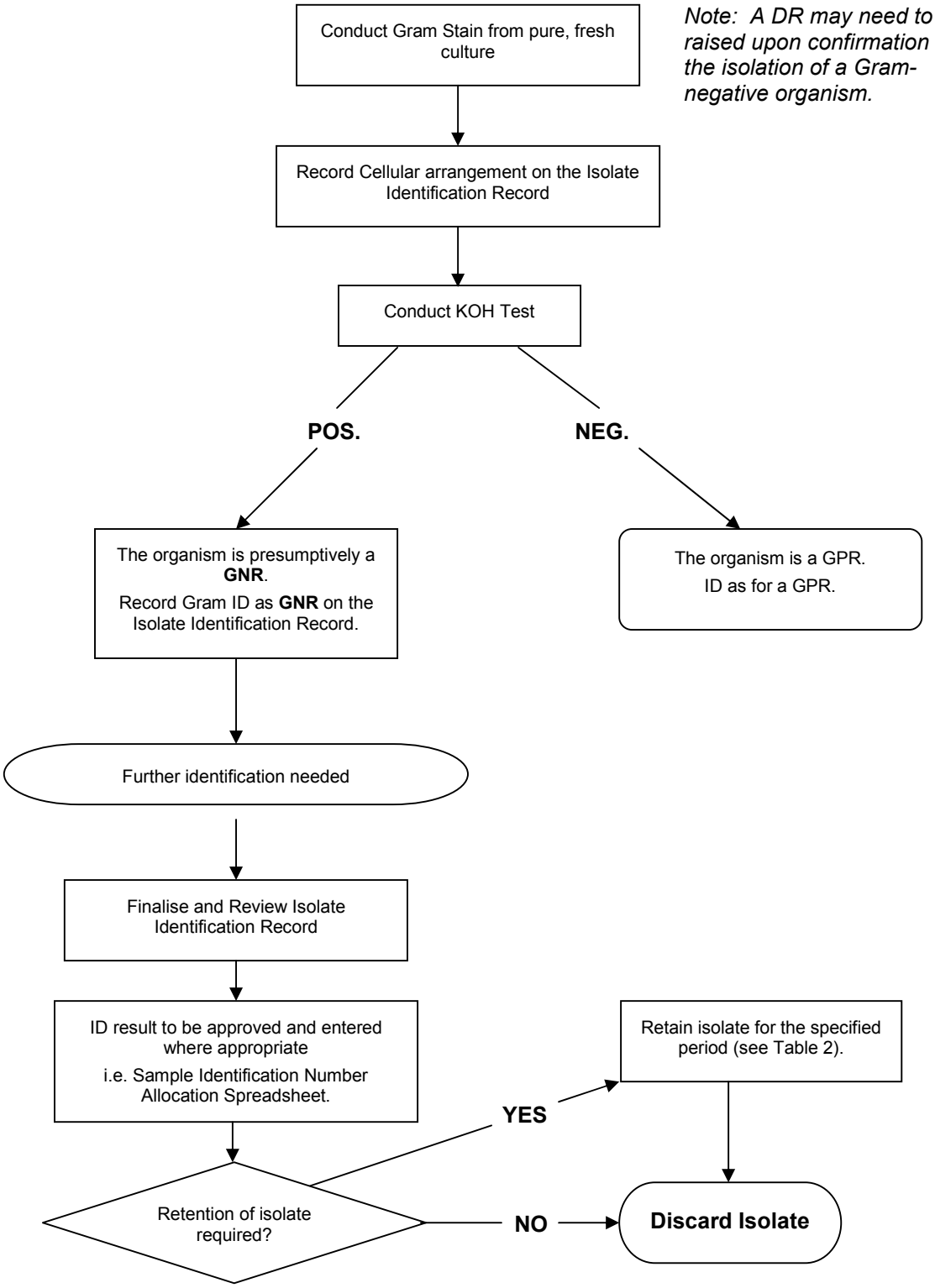
Rod/Bacillus - Bacilli are rod-shaped bacteria. Bacilli all divide in one plane producing a **bacillus**, **streptobacillus**, or **coccobacillus** arrangement (Fig 7B).

Pleomorphic rods of the genus Corynebacterium also divide in one plane, however they possess a characteristic v-shaped arrangement known as a **palisade** (Fig. 7B)**.** This is due to a snapping movement, which occurs immediately after cell division, which brings the cells into this arrangement.

Figure 7: Cellular Arrangements



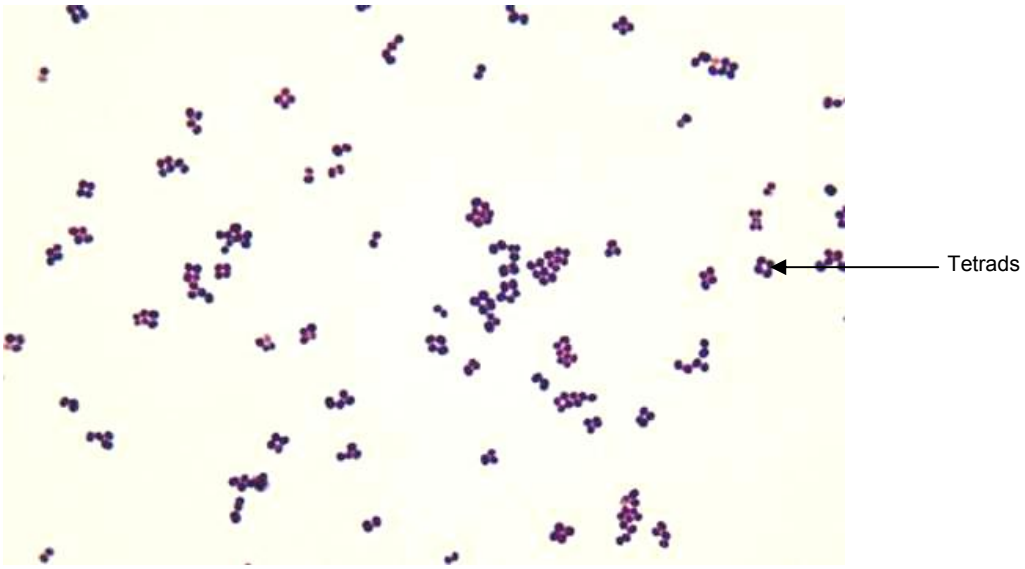
7.5. **ID Flowchart: Gram Negative Rods**



Note: A DR may need to be raised upon confirmation of the isolation of a Gram-negative organism.

7.7.3. *Micrococcus* species

Figure 09: Gram Stain of *Micrococcus* cells

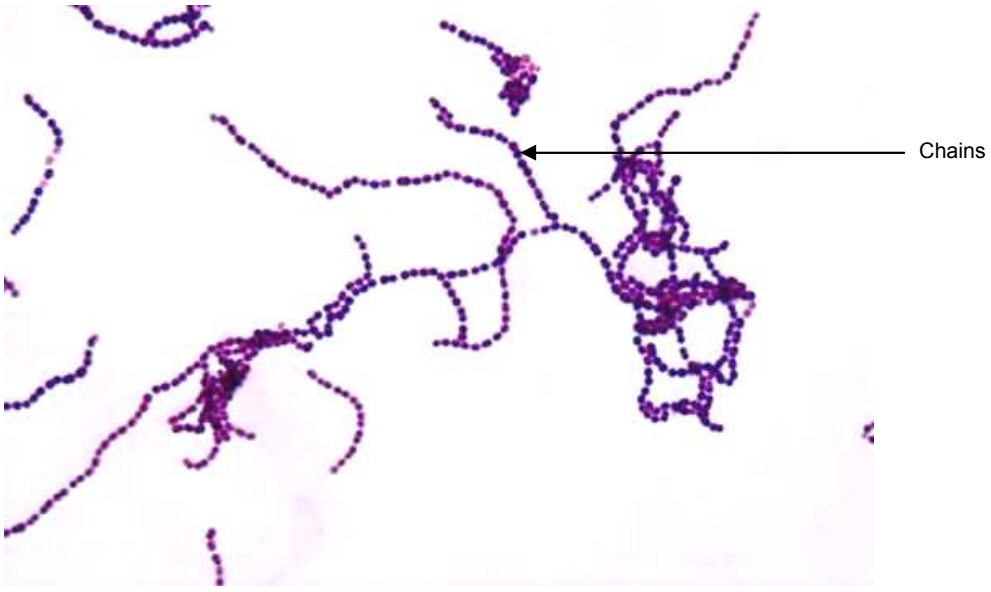


7.7.4. General Information

- *Micrococcus* species are **strictly aerobic**.
- *Micrococcus luteus* produces yellow colonies.
- Cells are large **Gram-positive cocci** arranged in tetrads.
- Catalase **positive**.
- Optimum growth temperature is between 30-37ºC.
- **Sources -** Environmental – Soil, Water; Human – Skin

7.7.5. Streptococcus species

Figure 10: Gram Stain of *Streptococcus* cells

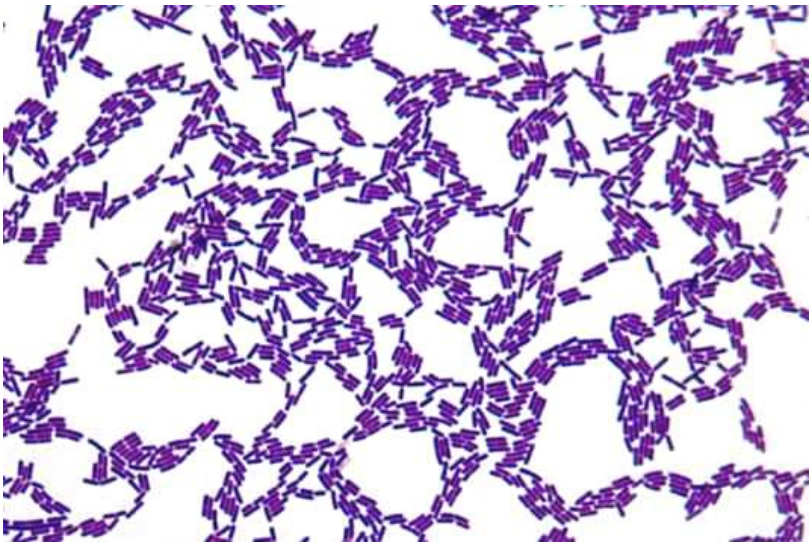


- **Sources -** Environmental – Soil, Contaminated Water; Human – Gastrointestinal tract, Female Genital Tract *(C.perfringens)*

7.9. Gram Positive Rods – Non-sporing

7.9.1. Lactobacillus species

Figure 13: Gram Stain of *Lactobacillus* cells



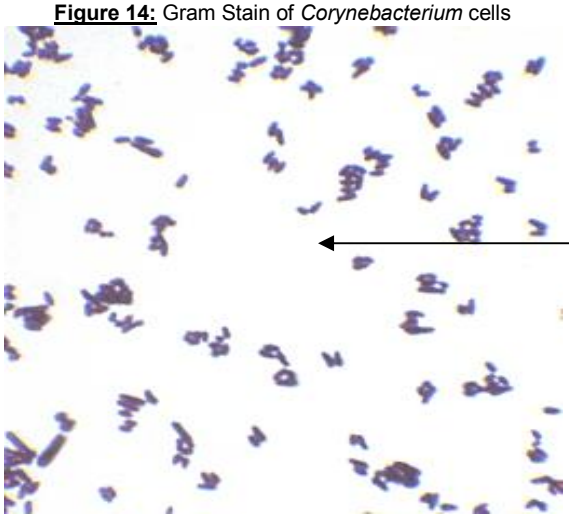
7.9.2. General Information

- **Gram-positive** large rods, non-spore forming, **anaerobic** or **microaerophilic**, occur singly or in pairs.
- Convert Lactose and other sugars to Lactic Acid.
- **Sources -** Normal flora of the following sites of Humans and Animals:

Mouth	Oropharynx	Gastrointestinal tract
Female Genital Tract		
Food – Dairy products		

7.9.3. *Corynebacterium* species

Figure 14: Gram Stain of *Corynebacterium* cells



Characteristic Palisade or "V" shape arrangement.

Cells pictured here have tapered ends, however ends may also appear club shaped.

7.10.5. Other commonly isolated **Non-fermenting Gram-negative rods** include:

- ***Achromobacter (Alcaligenes) xylosoxidans***
  Alcaligenes xylosoxidans was reclassified as *Achromobacter xylosoxidans* in 1998. It is both catalase- and oxidase positive.
- ***Alcaligenes* species**
  Colonies have a thin, spreading irregular edge. It is catalase negative, oxidase positive and motile.
- ***Brevundimonas* species**
  Brevundimonas vesicularis and *Brevundimonas diminuta* grow slowly on ordinary nutrient media. It forms a carotenoid pigment that produces yellow or orange colonies.
- ***Elizabethkingia* species**
  Elizabethkingia (formerly *Chryseobacterium*) *meningosepticum,* is the species of *Elizabethkingia* most often associated with serious infection. *E. meningosepticum* is non-motile and oxidase positive. *E. indologenes* is also non-motile and oxidase positive.
- ***Comamonas* species**
  It is motile, oxidase and catalase positive.
- ***Methylobacterium* species**
  The organism is oxidase positive and motile, but both of these characteristics may be weak. *Methylobacterium* species are Gram-negative but may stain poorly or show variable results. It has a characteristic microscopic appearance because individual cells contain large, non-staining vacuoles.
- ***Ochrobactrum* species**
  Colonies appear circular, low convex, smooth, and shining. Mucoid colonies may be produced on some media.
- ***Ralstonia* species**
  Ralstonia pickettii (formerly *Burkholderia pickettii*) is non-pigmented, oxidase-positive, and will grow at 41°C.
- ***Shewanella* species**
  Shewanella putrefaciens is oxidase-positive and motile.
- ***Sphingobacterium* species**
  They are oxidase-positive and non-motile. Colonies produce yellow pigment.
- ***Stenotrophomonas* species**
  Catalase positive and Oxidase negative.

