INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES

Improving The Quality of Life

PROTOCOL

Isolation and Identification of Bad Axillary Odors Producing Microorganism from Human Axillary Region

Laboratory : Indonesia International Institute for Life Sciences

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FOREWORD

This protocol is based on the bad axillary odors producing microorganism isolation and identification (*Corynebacterium spp.*, *Staphylococcus hominis, Staphylococcus spp.*, and *Micrococcus luteus*) from the human axillary region conducted at Indonesia International Institute for Life Sciences (i3L), Jakarta, Indonesia. The process is done to obtain the model microbial culture for in vitro antimicrobial activity testing of a deodorant product. The study was conducted by taking microbial samples from the human axillary region, selective media preparation, isolation through streak plating, identification of microorganism characteristics (morphological, biochemical, and molecular identification).

This protocol can be used as an example for those conducting similar studies that attempts for microbial isolation and identification of *Corynebacterium spp.*, *Staphylococcus spp.*, or *Micrococcus spp.* , or other microorganisms with adjustments made in accordance to the study conducted.

INTRODUCTION

The human axilla could release smelly and excessive axillary odor as a result of the metabolic activity of the human microflora and secretion of sweat and apocrine glands. Secretions of sweat and apocrine glands produce substances such as sodium chloride, fatty acid, urea, steroids, and proteins, which are metabolized by some microorganisms to produce volatile fatty acids and thioalcohols that are considered as bad odors (Semkova et al., 2015; Sorokowska, Sorokowski, & Szmajke, 2012).

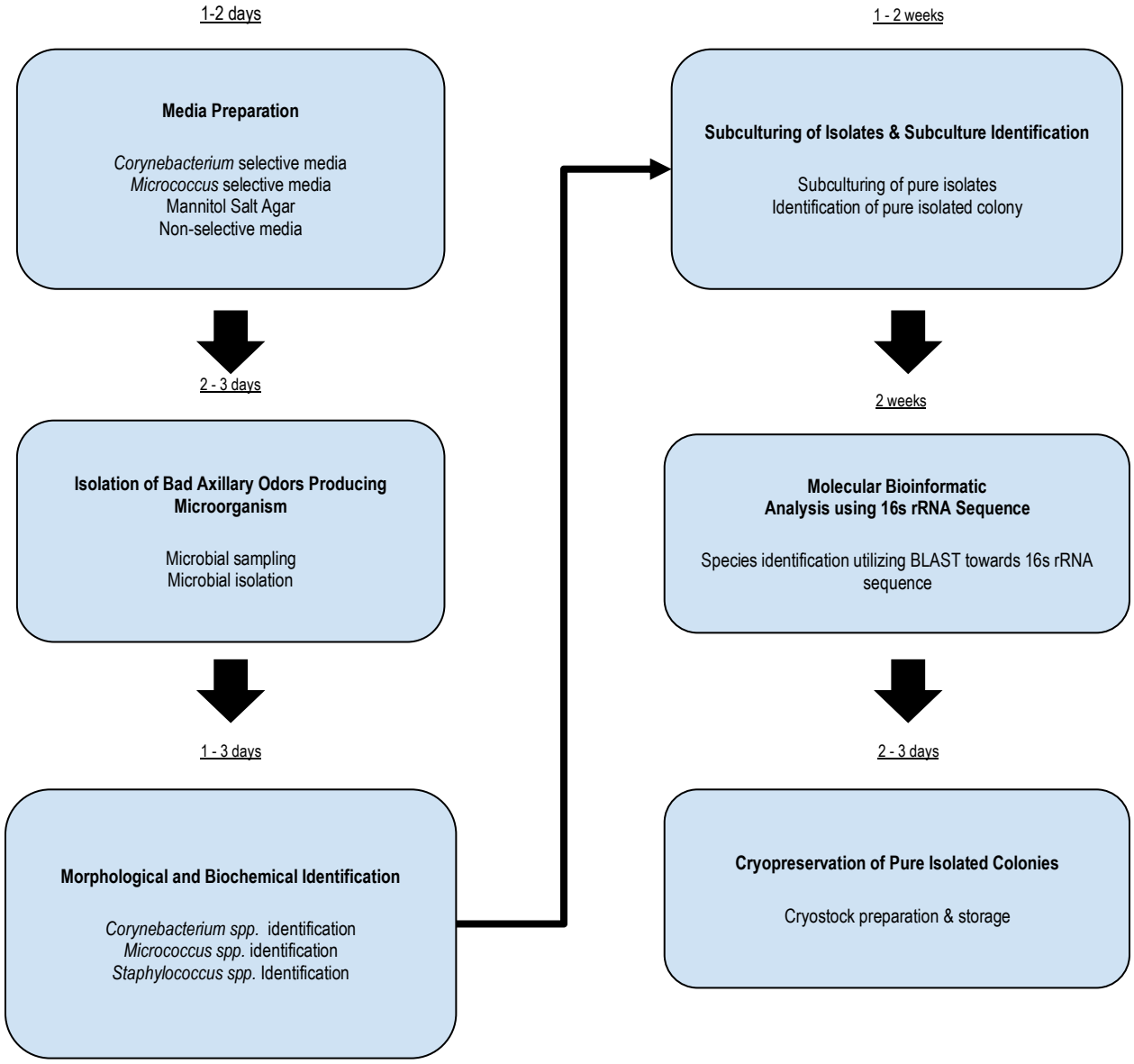
Microorganisms involved in bad axillary odor production fall to several genera: *Corynebacterium, Staphylococcus,* and *Micrococcus* (Taylor et al., 2003; Leyden et al., 1981; James et al., 2013). *Corynebacterium* groups are found to break down sterols to short-chain fatty acids, mainly 3-methyl-2-hexenoic acids, which are characterized with bad, hircine-like odor (James et al., 2013; James et al., 2004; Troccaz et al., 2015; Barzanty, Brune, & Tauch, 2011). *Staphylococcus* groups are found to produce diacetyl components, which have odors equivalent to the smell of carboxylic acid. *Staphylococcus hominis* is found as the strongest contributor towards axillary malodor as it produces a high concentration of thioalcohol, 3-methyl-3-sulfanylhexan-1-ol, which the production of this compound is insignificant in other *Staphylococcus* species (Troccaz et al., 2015; Rudden et al., 2020; Hara, Matsui, & Shimizu, 2014). *Micrococcus* genus is found to contribute towards malodor intensity; but shows low prevalence in the human axillary region (Taylor et al., 2003; James et al., 2013).

To reduce the amount of bad axillary odors producing microbes, a cosmetic product called deodorant is generally used. Deodorant acts as the antimicrobial agent that inhibits or kills the microorganisms contributing in bad axillary malodor production while enhancing the pleasantness of body odors (Darlenski & Fluhr, 2010). As a deodorant product should work in reducing bad axillary odor-producing microorganisms, antimicrobial testing of deodorant products is required to ensure the effectiveness, efficacy, and quality of the product.

Antimicrobial testing of deodorant products may require the use of lab grown culture of bad axillary odors producing microorganisms. This is where isolation and identification of bad axillary odors producing microorganisms from the human axilla play an important role, which is to obtain the pure culture of odors producing microorganisms for antimicrobial testing.

This module describes the procedure for isolation and identification of *Corynebacterium, Staphylococcus* (one *S. hominis* and one other *Staphylococcus*), and *Micrococcus*. Readers will be guided on how to conduct selective media making, colony morphology observation, microscopic observation with gram staining, biochemical observation such as oxidase test and anaerobic thioglycollate test. Readers will also learn how to subculture the identified microorganism to obtain the pure culture, and conduct molecular bioinformatic analysis of the pure culture using the 16s rRNA genetic sequence for sequencing by a third-party provider. Finally, readers will be guided on how to preserve the culture using a long term preservation method.

FLOWCHART


1-2 days
Media Preparation
Corynebacterium selective media
Micrococcus selective media
Mannitol Salt Agar
Non-selective media
2 - 3 days
Isolation of Bad Axillary Odors Producing Microorganism
Microbial sampling
Microbial isolation
1 - 3 days
Morphological and Biochemical Identification
Corynebacterium spp. identification
Micrococcus spp. identification
Staphylococcus spp. Identification
1 - 2 weeks
Subculturing of Isolates & Subculture Identification
Subculturing of pure isolates
Identification of pure isolated colony
2 weeks
Molecular Bioinformatic Analysis using 16s rRNA Sequence
Species identification utilizing BLAST towards 16s rRNA sequence
2 - 3 days
Cryopreservation of Pure Isolated Colonies
Cryostock preparation & storage

METHODOLOGY

Media Preparation of *Corynebacterium* Selective Media, *Micrococcus* Selective Media, Mannitol Salt Agar, and Non-selective Media

Background

One of the basic principles in microbiological isolation is through the choices of selective media. Selective media are usually prepared by formulating basal medium with antibiotics, salts, reducing sugars, growth factors, or other chemicals which are important to enhance the growth of desired microorganisms and to eliminate / inhibit the growth of most undesired microorganisms. *Corynebacterium* selective media is prepared by supplementing Tryptic Soy Agar (TSA) media with Tween 80 and antibiotic fosfomycin, which are used to enrich the growth of *Corynebacterium* species and to eliminate other microorganisms beside *Corynebacterium,* respectively. *Micrococcus* selective media is prepared using similar media with different supplementation, where antibiotic furazolidone is added to the agar media to eliminate non *Micrococcus* groups. Lastly, Mannitol Salt Agar is prepared for selecting *Staphylococcus* species due to its survival ability in high salt concentration.

Goal

To prepare selective agar media needed for selective bacterial isolation from human axillary region.

Materials

- Merck™ Tryptic Soy Agar (TSA) media powder
- Yeast extract
- Distilled water (Type III water)
- Fosfomycin
- Aluminum foil
- Tween 80
- Plastic wrap
- Petri dishes
- Furazolidone
- Dimethyl sulfoxide (DMSO)
- Mannitol Salt Agar (MSA)

Equipment

- Sterile micropipette tips 1000 µL
- Micropipette 1000 µL
- 500 ml Erlenmeyer Flask
- 250 ml Erlenmeyer Flask
- Hot plate
- Magnetic stirrer
- Autoclave
- Reaction tube
- 4°C refrigerator

Procedure

A. <u>*Corynebacterium* selective media preparation:</u>
   1. Weight 12 grams of TSA agar media powder and 0.9 grams of yeast extract, and transfer into a 500 mL flask containing 300 mL of distilled water.

2. Insert magnetic stirrer into the flask. Mix and boil the solution by using a hot plate.
3. Put off the flask from the hotplate when the mixture is already homogeneous. Remove the magnetic stirrer from the flask.
4. Seal the flask's cap with aluminum foil, autoclave for 15 mins in 121^{o}C.
5. While waiting for the autoclave, prepare 150 mg of antibiotic fosfomycin. Dissolve the fosfomycin with 7.5 mL sterile distilled water in a sterile reaction tube. Mix to form a homogeneous solution.
6. After the autoclave process is finished, cool the sterile media to approximately 45-50^{o}C. Aseptically, transfers 3 mL of Tween 80 and 7.5 mL of prepared fosfomycin solution into the flask. Mix well the sterile media solution by shaking the flask firmly.
7. Aseptically, pour approximately 10-20 mL of sterile media into a petri dish. Continue pouring to several petri dishes until all media in the flask is emptied.
8. After the agar media in petri dishes solidify, seal the agar media with plastic wrap and store in the refrigerator at 4^{o}C prior to use.

B. *Micrococcus* selective media preparation
1. Weight 12 grams of TSA agar media powder and 0.9 grams of yeast extract, and transfer into a 500 mL flask containing 300 mL of distilled water.
2. Insert magnetic stirrer into the flask. Mix and boil the solution by using a hot plate.
3. Put off the flask from the hotplate when the mixture is already homogeneous. Remove the magnetic stirrer from the flask.
4. Seal the flask's cap with aluminum foil, autoclave for 15 mins in 121^{o}C.
5. While waiting for the autoclave, prepare 120 mg of antibiotic furazolidone. Dissolve the furazolidone with 3 mL dimethyl sulfoxide (DMSO) in a sterile reaction tube. Mix to form a homogeneous solution.
6. After the autoclave process is finished, cool the sterile media to approximately 45-50^{o}C. Aseptically, transfers 2.25 mL of furazolidone solution into the flask. Mix well the sterile media solution by shaking the flask firmly.
7. Aseptically, pour approximately 10-20 mL of sterile media into a petri dish. Continue pouring to several petri dishes until all media in the flask is emptied.
8. After the agar media in petri dishes solidify, seal the agar media with plastic wrap and store in the refrigerator at 4^{o}C prior to use.

C. Mannitol Salt Agar preparation
1. Weight 16.67 grams of MSA agar media powder, and transfer into a 250 mL flask containing 150 mL of distilled water.
2. Insert magnetic stirrer into the flask. Mix and boil the solution by using a hot plate.
3. Put off the flask from the hotplate when the mixture is already homogeneous. Remove the magnetic stirrer from the flask.
4. Seal the flask's cap with aluminum foil, autoclave for 15 mins in 121^{o}C.
5. After the autoclave process is finished, cool the sterile media to approximately 45-50^{o}C.

6. Aseptically, pour approximately 10-20 mL of sterile media into a petri dish. Continue pouring to several petri dishes until all media in the flask is emptied.
7. After the agar media in petri dishes solidify, seal the agar media with plastic wrap and store in the refrigerator at 4^{o}C prior to use.

METHODOLOGY

Isolation of Bad Axillary Odors Producing Microorganism from Human Axilla

Background

To isolate and purify the microorganism of interest, the desired microorganism needs to be taken from a source and the isolation method has to be applied for separating the individual microorganisms from the microbiome community. Bad axillary odors producing microorganisms are microorganisms inhabiting the human axillary region and producing unpleasant smell, which consists of strong producing axillary odors: *Corynebacterium spp.* and *Staphylococcus hominis*, and weak producing axillary odors: *Micrococcus spp.* and other *Staphylococcus* group. The sample is taken from the human axillary region using a cotton swab that will be further administered in Phosphate Buffered Saline (PBS) solution to maintain the physiological condition of microorganisms. The sample is isolated by streaking a loop of microbial culture in PBS solution to selective agar media. Streak plating is performed to isolate individual microorganisms from the community since streaking helps in physical separation of microorganisms by distributing them throughout the agar. The distribution by streak plating allows individual bacteria cells to be isolated. The indication of bacteria cell can be observed by the formation of colonies that are produced during incubation process.

Goal

To isolate *Corynebacterium sp.*, *Staphylococcus hominis,* other *Staphylococcus sp.*, and *Micrococcus sp.*

Materials

- Sterile cotton swab
- Phosphate Buffered Saline (1X)
- Human's axillary region (axillary microbial sample)
- Prepared *Corynebacterium* selective medium
- Prepared *Micrococcus* selective medium
- Prepared Mannitol Salt Agar.
- Parafilm

Equipments

- 1.5 mL microcentrifuge tube
- 4^{o}C refrigerator
- Inoculation loop
- Bunsen burner
- 37^{o}C Incubator

Procedure

A. Microbial Sampling
   1. Dip sterile cotton swab in 1 mL of 1X PBS for 5 seconds.
   2. Obtain microbial samples by swabbing the subject's axillary region with wetted sterile cotton swab. Swab the axillary region for 45 seconds within the area shown in Figure 1.

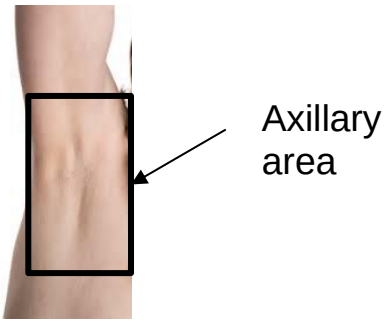


Figure 1. Region of axillary area for swabbing.

3. Put the sterile cotton swab containing the axillary microbial sample into a 1.5 mL microcentrifuge tube containing 1 mL of 1X PBS.
4. Store the sample in the refrigerator at 4°C prior to use.

B. Microbial Isolation

(Perform aseptic technique for all procedures below.)

1. Sterilize the inoculation loop with a bunsen burner.
2. Take a loopful of microbial samples stored in a 1.5 mL microcentrifuge tube.
3. Streak the microbial samples to *Corynebacterium* selective medium, *Micrococcus* selective medium, mannitol salt agar, and non-selective medium using four quadrant streak method (Figure 2).

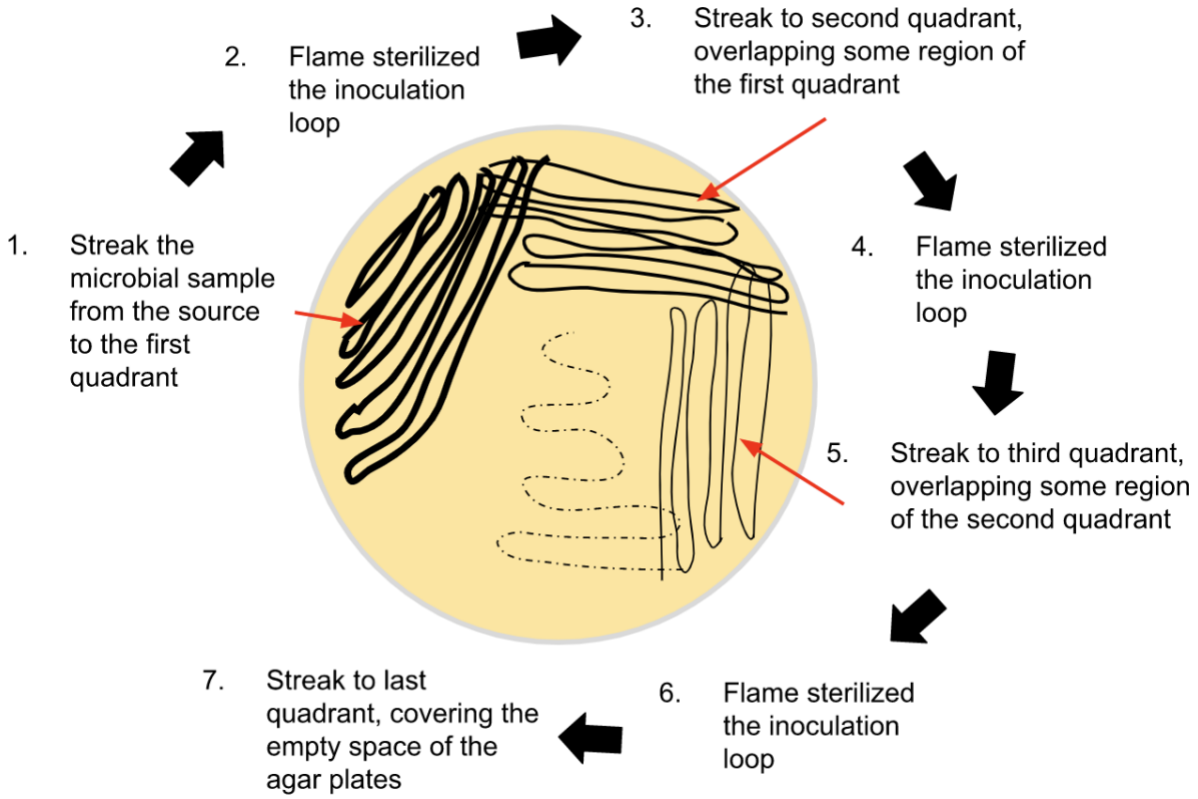


Figure 2. Procedure for streak plate method.

4. Duplicate the isolation process.
5. Transfer 1 mL of PBS into another sterile 1.5 mL microcentrifuge tube. Streak the sterile PBS sample into all the agar plates as aseptic technique control.
6. Seal all the plates with parafilm. Make sure the parafilm covers all the openings to prevent contamination from outside sources.
7. Incubate all plates at 37^{o}C incubator for 1 - 3 days prior to the cultural observation and identification.

METHODOLOGY

Morphological and Biochemical Identification of Bad Axillary Odor Producing Microorganism

Background

To isolate the microorganism of interest, identification is the essential step to determine the physicochemical characteristics of the microorganism since different groups of microorganisms have different characteristics. Microorganisms can be distinguished physicochemically by colony morphology (shape, elevation, margin, color, texture, and opacity), microscopic morphology (gram type, shape, arrangement, and motility) and biochemical (sugar fermentation, enzymatic activity, etc). However, the physicochemical test only produces presumptive identification of species / species group since the result does not represent the genotypic characteristics that are highly accurate with the name of the species.

Goal

To obtain the presumptive species / species groups *Corynebacterium sp*., *Staphylococcus hominis*, other *Staphylococcus sp.,* and *Micrococcus sp.* by identifying the morphological and biochemical characteristics.

Materials

- Incubated Microbial Sample in *Corynebacterium* Selective Media, *Micrococcus* Selective Media, and Mannitol Salt Agar
- Crystal Violet
- Lugol's Iodine
- Decolorizing Agent (Ethanol/Acetone)
- Safranin
- Immersion oil
- Distilled water
- Parafilm
- Bactident™ Oxidase test strip
- *Pseudomonas aeruginosa* culture in TSA
- *Staphylococcus aureus* culture in TSA
- Tryptic Soy Broth
- Thioglycollate Broth
- 0.5 McFarland standard

Equipments

- Microscope
- Glass slide
- Metal inoculation loop
- Wooden stick applicator
- Sterile micropipette tips 1000 µL
- Sterile micropipette tips 200 uL
- Micropipette 1000 uL
- Micropipette 200 uL
- Falcon tube
- Bunsen burner
- 4°C Refrigerator
- Orbital shaker incubator
- Black paper strips

Procedure

A. Identification of *Corynebacterium* species from *Corynebacterium* Selective Media

1. Take out the incubated microbial sample in *Corynebacterium* Selective Media
2. Observe the colony characteristics (color, shape, elevation, edge, and size). *Corynebacterium* colony should appear white/grayish color, circular shape, raised elevation, entire edge, and small size (0.2 mm - 1 mm). Take pictures whenever necessary.
3. Observe the agar plates containing sterile PBS. Aseptic technique is successfully followed if the agar plates do not show any colonies.
4. Pick up one colony corresponding to the colony characteristic of *Corynebacterium* for gram staining using the procedure in Figure 3.

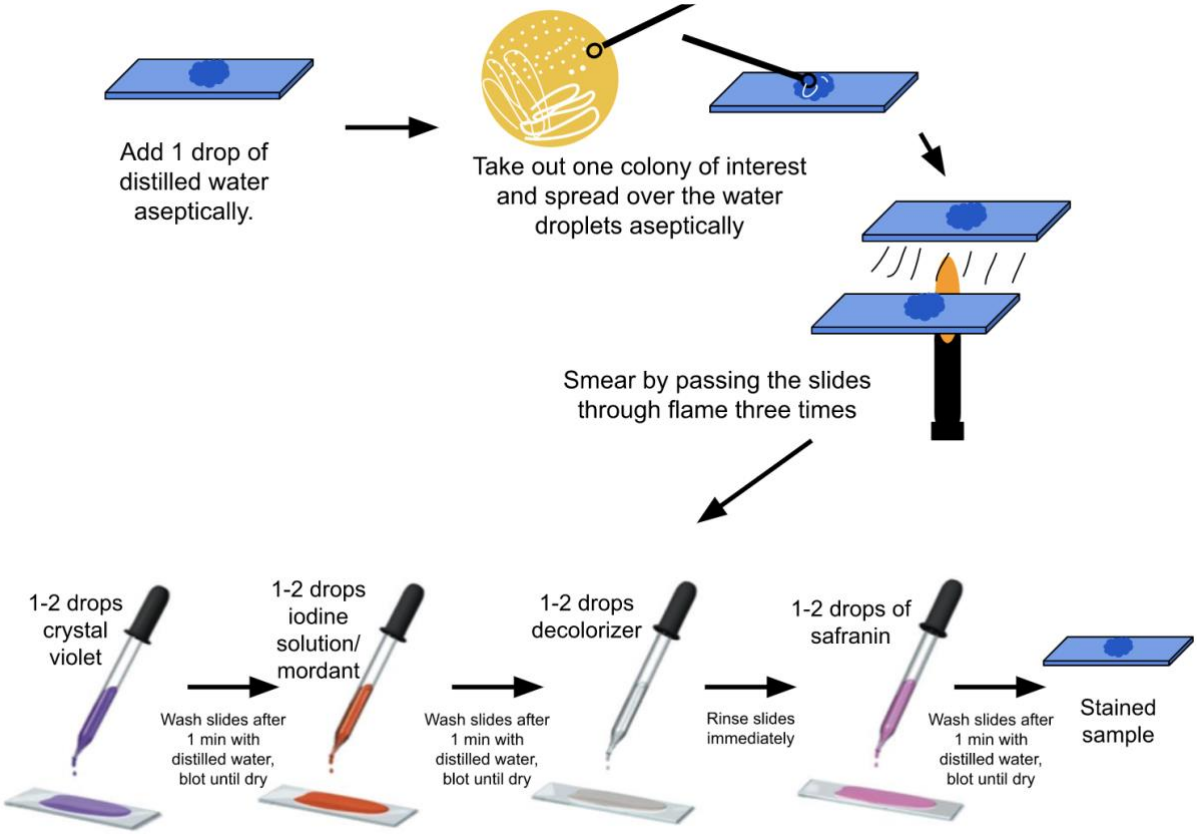


Figure 3. Procedure for gram staining

5. Observe the gram stained colony using a microscope with 1000x magnification (before observing samples in 1000x, gradually observe the microorganisms in 40x, 100x, and 400x magnification to adjust the focus). Apply immersion oil before observing the sample in 1000x magnification.
6. Observe the microscopic morphology of *Corynebacterium* species. *Corynebacterium* should produce rod shaped bacterium with tapered/clubbed ends, with purple gram stain color (gram positive). Take pictures whenever necessary.
7. After the identification process, seal the plate with parafilm and store in a 4^{o}C refrigerator prior subculturing.

B. Identification of *Micrococcus* species from *Micrococcus* Selective Media

1. Take out the incubated microbial sample in *Micrococcus* Selective Media.
2. Observe the colony characteristics (color, shape, elevation, edge, and size). *Micrococcus* colonies should appear yellow in color, circular shape, convex elevation, entire edge, smooth appearance, and large size (3 - 5 cm). Take pictures whenever necessary.
3. Pick up one colony corresponding to the colony characteristic of *Micrococcus* for oxidase test using wooden stick applicator (or other non-metal labwares).
4. Take out one Bactident® oxidase test paper strip. Rub the sample towards the reaction zone (Figure 4).

Figure 4. Oxidase test strips

5. Observe the changes of color within 20 - 60 seconds. Changes of color to dark blue to purple showing oxidase positive results. *Micrococcus* colonies should show positive results.
6. Apply a test for *Pseudomonas aeruginosa* (oxidase positive) and *Staphylococcus aureus* (oxidase negative) as oxidase test control.
7. Pick up one colony corresponding to the colony characteristic of *Micrococcus* for gram staining using the procedure in Figure 3 above.
8. Observe the gram stained colony using a microscope with 1000x magnification (before observing samples in 1000x, gradually observe the microorganisms in 40x, 100x, and 400x magnification to adjust the focus). Apply immersion oil before observing the sample in 1000x magnification.
9. Observe the microscopic morphology of *Micrococcus* species. *Micrococcus* should be cocci shaped bacterium with tetrad arrangement with purple gram stain color (gram positive). Take pictures whenever necessary.
10. After the identification process, seal the plate with parafilm and store in a 4^{o}C refrigerator prior subculturing.

C. Identification of *Staphylococcus* species (*S. hominis* and other *Staphylococcus*) from *Staphylococcus* Selective Media

1. Take out the incubated microbial sample in Mannitol Salt Agar
2. Observe the colony characteristics (color, shape, elevation, edge, size, and mannitol reaction). Bad odors producing Staphylococcus are mannitol negative, choose the mannitol negative microorganism for further analysis.
3. For each colony characteristics, pick up several colonies (3 - 5 colonies) for oxidase test. Negative results should appear as a correct identification for *Staphylococcus* species.
4. Pick up the colonies from an agar plate that shows a negative result of oxidase test for gram stain using the procedure from Figure 3 above.
5. Observe the gram stained colony using a microscope with 1000x magnification (before observing samples in 1000x, gradually observe the microorganisms in 40x, 100x, and 400x magnification to adjust the focus). Apply immersion oil before observing the sample in 1000x magnification.
6. Observe the microscopic morphology of *Staphylococcus* species. *Staphylococcus* should appear as gram positive, cocci shape, and irregular clusters. For *Staphylococcus hominis,* clusters dominated with tetrad arrangement should be observed. For other *Staphylococcus*, clusters are dominated with irregular arrangement. Take pictures whenever necessary.
7. For colonies that have been identified microscopically, subculture them to a prepared 5 mL sterile Tryptic Soy Broth media in a falcon tube aseptically.
8. Inoculate *Pseudomonas aeruginosa* and *Staphylococcus aureus* to other 5 mL Tryptic Soy Broth media separately.
9. Incubate all cultures for approximately 12 hours at 37°C, 200 rpm in an orbital shaker incubator.
10. Adjust the culture density to 0.5 McFarland Standard in a falcon tube by comparing the turbidity visually with the help of black and white stripe paper (Figure 5)

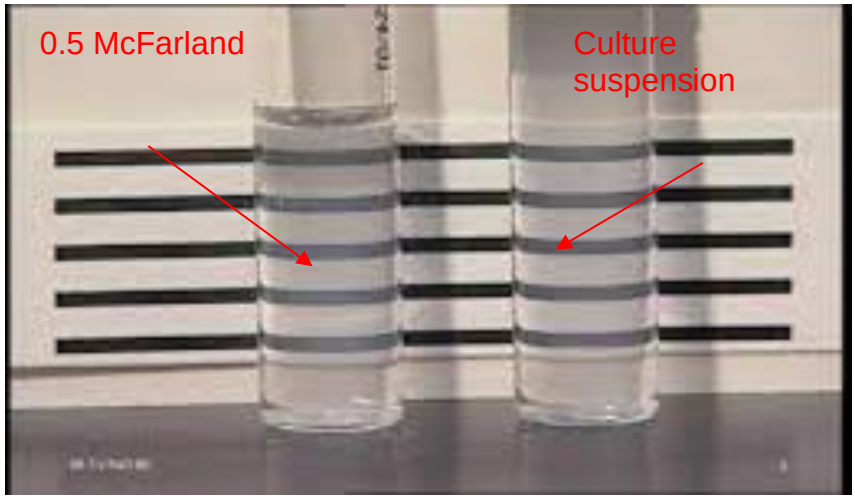


Figure 5. Example of turbidity comparison with 0.5 McFarland Standard

11. Transfer 20 µL of culture to a sterile 5 mL Thioglycollate Broth for anaerobic growth testing.
12. Incubate the inoculum overnight at 37°C, 200 rpm in an orbital shaker incubator.

13. Compare the anaerobic growth pattern and turbidity of subcultured *Staphylococcus* with *Pseudomonas aeruginosa* and *Staphylococcus aureus* using black-white striped paper. *S. hominis* should produce lower microbial turbidity comparative with *Pseudomonas aeruginosa* (negative growth in anaerobic thioglycollate). Meanwhile, other *Staphylococcus* produce higher microbial turbidity comparative with *Staphylococcus aureus* (positive growth in anaerobic thioglycollate).
14. After the identification process, seal the plate with parafilm and store in a 4^{o}C refrigerator prior subculturing.

METHODOLOGY

Subculturing of Isolated Colonies to Another Media & Identification of Subcultures

Background

Subculturing is the process of purifying the microorganism from the mixture of microorganisms. This method is attempted to allow only individual microorganisms existing in agar plates prior to molecular identification and bacterial culture stock / collection preparation. Furthermore, subculturing can be used to maintain the microorganism for a short term period.

Goal

To subculture and purify the individual bacteria colonies of *Corynebacterium sp*., *Staphylococcus hominis*, other *Staphylococcus sp*., and *Micrococcus sp.*

Materials

- *Corynebacterium* Selective Media, *Micrococcus* Selective Media, and Mannitol Salt Agar in petri dish
- Culture plates containing presumptively identified colonies of *Corynebacterium spp., Staphylococcus spp.* (*S. hominis* and non *S. hominis*), and *Micrococcus spp.*
- Parafilm
- Distilled water
- Crystal Violet
- Lugol's Iodine
- Decolorizer (Ethanol/Acetone)
- Safranin

Equipments

- Inoculation loop
- 37^{o}C Incubator
- Microscope
- Glass slide

Procedure

1. For each identified colony, subculture the single colony to a new medium by using four-way streaking method aseptically (Session 3, Figure 2). The medium for subculture of each identified colony is provided in Table 1 below.

Table 1. Medium for subculture of each presumptive microorganism.

Presumptive Colony	Medium for Subculture
Corynebacterium spp.	*Corynebacterium* Selective Media
Micrococcus spp.	*Micrococcus* Selective Media
Staphylococcus spp.	Mannitol Salt Agar

2. Seal the petri dishes with parafilm.
3. Incubate all agar plates at 37^{o}C incubator for 1 - 2 days.
4. Observe the colony characteristics and microscopic morphology of each sub-cultured isolates. Consistent result of colony characteristic and microscopic morphology without growth of other colonies showing the retrieval of pure isolated colonies.

METHODOLOGY

Molecular Bioinformatic Analysis of Pure Isolated Colonies using 16s rRNA Sequence

Background

Molecular analysis using the bioinformatic tools have been used widely to determine the name of the species by taking advantage of genomic characteristics from individual cells. In bacteria cells, 16s rRNA sequence has been applied as a golden standard for species identification since this gene presence in most of the bacterial cells, and the evolution of this gene corresponds with the evolution of the microorganism. This means that every species has an unique 16s rRNA sequence. Furthermore, this identification is more simple, rapid, and inexpensive in comparison with the other molecular identification methods.

Bioinformatic tools utilized to identify the sequence is Basic Local Alignment Search Tool (BLAST) by NCBI. This tool can be used to compare the DNA sequence from the sequencing result with the reference sequence in the database to determine the name of the species. The species name of the sequence is determined from comparing the highest percentage identity, lowest expected value, and highest query coverage of the sequence with the reference sequence.

Goal

To identify the species name from pure isolated colonies containing presumptive *Corynebacterium sp*., *Staphylococcus hominis*, other *Staphylococcus sp.,* and *Micrococcus sp.*

Materials

- Pure isolated colonies of *Corynebacterium spp.*, *Staphylococcus spp.* (*S. hominis* & non *S. hominis*), and *Micrococcus spp* in agar plate
- FASTA code of 16s rRNA sequence from isolated colonies

Equipments

- Internet connection
- Mobile devices with browser

Procedure

1. Send the pure isolated colonies in agar plate to a third party company that provides services for 16s rRNA sanger sequencing / DNA barcoding. The company will conduct DNA isolation and purification, Polymerase Chain Reaction using 16s rRNA forward and reverse primer, and DNA sequencing.
2. After the sequencing process, the company will give the sequence alignment in FASTA code format.
3. Identify the species name by using the Basic Local Alignment Search Tool (BLAST) program provided by NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi)
4. Select nucleotide BLAST (BLASTn) tool.
5. Input the FASTA code format given for each isolates.

6. Under the Choose Search Set tab, select “rRNA/ITS database” for Database, and select “16s rRNA sequence for Bacteria and Archaea” in the dropdown menu. Furthermore, tick Yes in the exclude section for “Uncultured/environmental sample sequences”
7. In the Program Selection tab, choose “highly similar sequences (megablast)”.
8. Click BLAST and wait for several minutes.
9. Species appear on the Top BLAST Hits (highest percentage identity and lowest E-value), showing the identity of the isolates obtained. Record the result as the confirmed name of the isolate.

METHODOLOGY

Cryopreservation of Pure Isolated Culture

Background

Cryopreservation is a long term preservation method where the stock of microorganisms are kept in their dormant state (inactive), and free from any possible genetic changes or contamination for future use. This step is important as having stored microorganisms could help in shortening the microbiology research duration by removing the initial step of microbial isolation and identification. The cryopreservation of microorganisms utilizes a very low temperature for this inactivation since a low temperature will inhibit the kinetic activity of biological cells. Storing the sample in very low temperature requires the use of a cryoprotectant agent, glycerol, which acts to prevent the ice crystal formation that may damage the biological cells.

Goal

To store pure cultures of *Corynebacterium spp.*, *Staphylococcus spp.* (*S. hominis* & non *S. hominis*), and *Micrococcus spp.* for a long term using cryopreservation method.

Materials

- Pure isolated colonies of *Corynebacterium spp.*, *Staphylococcus spp.* (*S. hominis* & non *S. hominis*), and *Micrococcus spp.* in agar plate
- Tryptic soy broth
- 50% glycerol stock
- Parafilm

Equipments

- Falcon tubes
- 1.5mL cryovials
- Inoculation loop
- Sterile micropipette tips 1000 µL
- 37^{o}C incubator
- Sterile micropipette tips 200 µL
- Micropipette 1000 µL
- Micropipette 200 µL
- -80^{o}C freezer
- Cryovial storage box.

Procedure

1. Inoculate the pure isolated colony into 5 mL of prepared Tryptic Soy Broth in sterile falcon tube aseptically.
2. Seal the falcon tube with a screw cap. Cover the cap with parafilm
3. Incubate the inoculated pure culture overnight at the 37^{o}C incubator.
4. Transfer 0.5 mL of the incubated pure culture to 1.5 mL cryovial aseptically.
5. Add 0.5 mL of 50% glycerol stock to the 1.5 mL cryovial containing pure culture aseptically. Mix by resuspension.
6. Create several stocks of pure cultures as a preventive approach (in case one or some vials fails to retrieve the species after cryopreservation).

7. Store the pure culture stocks in cryovials in the -80^{o}C freezer.

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