Ministry of Higher Education and Scientific Research
University of Baghdad
College of Science
Department of Biology

Pathogenic Bacteria-Practical

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المرحلة الرابعة ـ الدراستين الصباحية والمسائية

الفصل الدراسي الاول

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CONTINGENCY PLANS

Lab One

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Risk Groups

Microorganisms are classified into Risk Groups according to the degree of risk of infectivity, pathogenicity, the availability of preventive measures and effective treatments, and potential damage to the environment. Risk Groups correlate to, but do not always equate with, biological safety levels. The table below describes the four Risk Groups with examples of organisms.

Table 1: Risk groups

Risk Group	Description	Examples
1	A microorganism that is unlikely to cause human or animal disease (no or low individual and community risk)	• *Bacillus subtilis*
2	Agents that are associated with human disease which is rarely serious or for which preventive and therapeutic interventions are often available (moderate individual risk, low community risk)	• *Salmonella enterica* sv. Typhimurium • *Pseudomonas aeruginosa* • Pathogenic *E. coli*
3	Agents that are associated with serious or lethal human disease for which preventive or therapeutic interventions may be available (high individual risk but low community risk)	• *Yersinia pestis* • *Francisella tularensis*
4	Agents that are likely to cause serious or lethal human disease for which preventive or therapeutic intervention are not usually available (high individual risk and high community risk)	• Ebola virus

Contingency plans and emergency procedures

Every laboratory that works with infective microorganisms should institute safety precautions appropriate to the hazard of the organisms and the animals being handled.

A written contingency plan for dealing with laboratory and animal facility accidents is a necessity in any facility that works with or stores Risk Group 3 or 4 microorganisms. National and/or local health authorities should be involved in the development of the emergency preparedness plan.

Contingency plan

The contingency plan should provide operational procedures for:

1. Precautions against natural disasters, e.g. fire, flood, earthquake and explosion
2. Biohazard risk assessment
3. Incident-exposure management and decontamination
4. Emergency evacuation of people and animals from the premises
5. Emergency medical treatment of exposed and injured persons
6. Medical surveillance of exposed persons
7. Clinical management of exposed persons
8. Epidemiological investigation
9. Post-incident continuation of operations.

In the development of this plan the following items should be considered for inclusion:

1. Identification of high-risk organisms.
2. Location of high-risk areas.
3. Identification of at-risk personnel and populations.
4. Identification of responsible personnel and their duties.
5. Lists of treatment and isolation facilities that can receive exposed or infected persons.
6. Transport of exposed or infected persons.
7. Lists of sources of immune serum, vaccines, drugs, special equipment and supplies.
8. Provision of emergency equipment.

Emergency procedures for microbiological laboratories

1. Puncture wounds, cuts and abrasions

The affected individual should remove protective clothing, wash the hands and any affected area(s), apply an appropriate skin disinfectant, and seek medical attention as necessary. The cause of the wound and the organisms involved should be reported, and appropriate and complete medical records kept.

2. Ingestion of potentially infectious material

Protective clothing should be removed and medical attention sought. Identification of the material ingested and circumstances of the incident should be reported, and appropriate and complete medical records kept.

3. Potentially infectious aerosol release

All persons should immediately vacate the affected area and any exposed persons should be referred for medical advice. The laboratory supervisor and the biosafety officer should be informed at once. No one should enter the room for an appropriate amount of time (e.g. 1 h), to allow aerosols to be carried away and heavier particles to settle. If the laboratory does not have a central air exhaust system, entrance should be delayed (e.g. for 24 h).

Signs should be posted indicating that entry is forbidden. After the appropriate time, decontamination should proceed, supervised by the biosafety officer. Appropriate protective clothing and respiratory protection should be worn.

4. Broken containers and spilled infectious substances

Broken containers contaminated with infectious substances and spilled infectious substances should be covered with a cloth or paper towels. Disinfectant should then be poured over these and left for the appropriate amount of time. The cloth or paper towels and the broken material can then be

cleared away; glass fragments should be handled with forceps. The contaminated area should then be swabbed with disinfectant. If dustpans are used to clear away the broken material, they should be autoclaved or placed in an effective disinfectant. Cloths, paper towels and swabs used for cleaning up should be placed in a contaminated-waste container. Gloves should be worn for all these procedures.

If laboratory forms or other printed or written matter are contaminated, the information should be copied onto another form and the original discarded into the contaminated-waste container.

5. Breakage of tubes containing potentially infectious material in centrifuges

If a breakage occurs or is suspected while the machine is running, the motor should be switched off and the machine left closed (e.g. for 30 min) to allow settling. If a breakage is discovered after the machine has stopped, the lid should be replaced immediately and left closed (e.g. for 30 min). In both instances, the biosafety officer should be informed.

Strong (e.g. thick rubber) gloves, covered if necessary with suitable disposable gloves, should be worn for all subsequent operations. Forceps, or cotton held in the forceps, should be used to retrieve glass debris.

All broken tubes, glass fragments, buckets, trunnions and the rotor should be placed in a noncorrosive disinfectant known to be active against the organisms concerned. Unbroken, capped tubes may be placed in disinfectant in a separate container and recovered.

The centrifuge bowl should be swabbed with the same disinfectant, at the appropriate dilution, and then swabbed again, washed with water and dried. All materials used in the clean-up should be treated as infectious waste.

Lab 2

Family: Enterobacteriaceae (*Escherichia coli* and *Klebsiella pneumoniae*)

1. *Escherichia coli*: Gram negative coccobacilli or bacilli, non-spore former, motile, facultative anaerobes.
2. *Klebsiella pneumoniae*: Gram negative bacilli, capsulated, the polysaccharide is very thick (mucoid appearance of the colonies)

Lab diagnostic tests

1- Gram stain.

2- Growth on MacConkey agar

Selective: Bile salts and crystal violet inhibit most gram-positive organisms and permit growth of gram-negative rods.

Differential: Lactose serves as the sole carbohydrate. Lactose fermenters produce pink or red colonies, may be precipitated bile salts may surround colonies. Non–lactose fermenters appear colourless or transparent.

This colour differentiation is due to the presence of neutral red indicator (yellow in alkaline pH and pink in acid pH).

3- Growth on Eosin Methylene Blue (EMB)

Selective for Enterobacteriaceae (contain and methylene blue inhibit Gram positive)

Differential for *E. coli* (green metallic sheen)

Lactose fermentation ----------> Acid + Eosin Y -----------> green metallic sheen

4- Growth on Triple Sugar Iron Agar (TSI).

Contains glucose, sucrose, and lactose. Sucrose and lactose are present in 10 times the quantity of the glucose; phenol red is the pH indicator. Turns to yellow when sugars are fermented because of drop in pH. Sodium thiosulfate plus ferric ammonium sulfate as H_2S indicator.

Acid/acid (A/A): Glucose and lactose and/or sucrose (or both) fermentation.

Gas bubbles: Production of gas (CO_2).

Visible air breaks or pockets in agar.

Black precipitate: H_2S.

Na – thiosulfate + $H_2 \rightarrow H_2S$

$H_2S + FeSO_4 \rightarrow FeS$ Black precipitate

Alkaline/acid (K/A): Glucose fermentation but not lactose or sucrose.

Alkaline/alkaline (K/K): No fermentation of dextrose, lactose, or sucrose.

5 - IMViC Test

Certain biochemical tests include Indole, Methyl red, Voges Proskauer, and Citrate utilization are known by the acronym IMViC.

a. Indole test: deamination of tryptophane to pyruvic acid

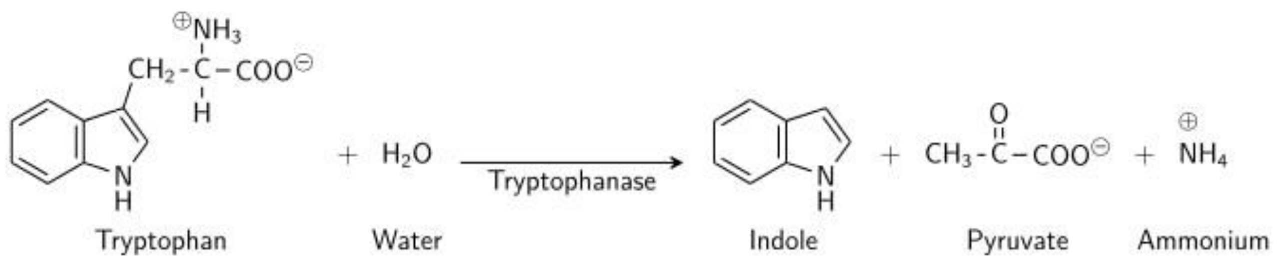
Purpose

This test is used to identify organisms that produce the enzyme tryptophanase.

Principle

The test is used to determine an organism's ability to hydrolyse tryptophan to form the compound indole. Tryptophan is present in

casein and animal protein. Bacteria with tryptophanase are capable of hydrolysing tryptophan to pyruvate, ammonia, and indole. Kovac's reagent (dimethylamine-benzaldehyde and hydrochloride), when added to the broth culture, reacts with the indole, producing a red colour. An alternative method uses Ehrlich's reagent. Ehrlich's reagent has the same chemicals as the Kovac preparation, but it also contains absolute ethyl alcohol, making it flammable. Ehrlich's reagent is more sensitive for detecting small amounts of indole.



Procedure

Media: Casein peptone (10 g), NaCl (5 g), tryptophan (10 g), per 1000 mL.

Method

1. Inoculate tryptophane broth with 1 drop from a 24-hour brain-heart infusion broth culture.
2. Incubate at 35°-37°C in ambient air for 48 hours.
3. Add 0.5 mL of Kovac's reagent to the broth culture.

Expected Results

Positive: Pink- to wine-colored ring after addition of appropriate reagent.

Negative: No colour change after addition of the appropriate reagent.

b. Methyl red / Voges - Proskauer

Purpose

The combination test methyl red (MR) and Voges-Proskauer (VP) differentiates members of the Enterobacteriaceae family. MR: glucose full fermentation. VP: glucose partial fermentation.

Principle

This test is used to determine the ability of an organism to produce and maintain stable acid end products from glucose fermentation, to overcome the buffering capacity of the system, and to determine the ability of some organisms to produce neutral end products (e.g., 2,3-butanediol or acetoin) from glucose fermentation. The methyl red detects mixed acid fermentation (pyruvic acid + lactic acid + formic + Succinic + acetic) that lowers the pH of the broth.

The MR indicator is added after incubation. Methyl red is red at pH 4.4 and yellow at pH 6.2. A clear red is a positive result; yellow is a negative result; and various shades of orange are negative or inconclusive.

The VP detects the organism's ability to convert the acid products to acetoin and 2,3-butanediol. Organisms capable of using the VP pathway produce a smaller amount of acid during glucose fermentation and therefore do not produce a colour change when the methyl red indicator is added. A secondary reagent is added, alpha-naphthol, followed by potassium hydroxide (KOH); a positive test result is indicated by a red colour complex.

Media:

Peptic digest of animal tissue (3.5 g), pancreatic digest of casein (3.5 g), dextrose (5 g), KPO_4 (5 g), per 1000 mL, pH 6.9.

Method

1. Inoculate MRVP broth with 1 drop from a 24-hour brain-heart infusion broth culture.
2. Incubate at 35°-37°C for a minimum of 48 hours in ambient air. Tests should not be made with cultures incubated less than 48 hours, because the end products build up to detectable levels over time. If results are equivocal at 48 hours, repeat the tests with cultures incubated at 35°-37°C for 4 to 5 days in ambient air; in such instances, duplicate tests should be incubated at 25°C.
3. Split broth into aliquots for MR test and VP test.

A. Methyl Red Test

1. Add 5 or 6 drops of methyl red reagent per 5 mL of broth.
2. Read reaction immediately.

Expected Results

Positive: Bright red color, indicative of mixed acid fermentation.

Weakly positive: Red-orange color.

Negative: Yellow color.

B. Voges-Proskauer Test (Barritt's Method) for Gram-Negative Rods

1. Add 0.6 mL (6 drops) of solution A (alpha-naphthol) and 0.2 mL (2 drops) of solution B (40% KOH) to 1 mL of MRVP broth.
2. Shake well after addition of each reagent.
3. Observe for 5 minutes.

Expected Results

Positive: Red color, indicative of acetoin production.

Negative: Yellow color.

Limitations

The MR test should not be read before 48 hours, because some organisms will not have produced enough products from the fermentation of glucose. MR-negative organisms may also not have had sufficient time to convert those products and will appear MR positive. MR-VP testing should be used in conjunction with other confirmatory tests to differentiate organisms among the Enterobacteriaceae.

c. Citrate utilization

Purpose

The purpose of this test is to identify organisms capable of using sodium citrate as the sole carbon source and inorganic ammonium salts as the sole nitrogen source. The test is used to differentiate Enterobacteriaceae from other gram-negative rods.

Principle

Bacteria that can grow on this medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. Pyruvate can then enter the organism's metabolic cycle for the production of energy. Bacteria capable of growth in this medium use the citrate and convert ammonium phosphate to ammonia and ammonium hydroxide, creating an alkaline pH. The pH change turns the bromothymol blue indicator from green to blue.
Media (Simmon's citrate): $NH_4H_2PO_4$ (1 g), K_2HPO_4 (1 g), NaCl (5 g), sodium citrate (2 g), $MgSO_4$ (0.2 g), agar (15 g), bromothymol blue (0.08 g), per 1000 mL, pH 6.9.

Method

1. Inoculate Simmon's citrate agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old. Do not inoculate from a broth culture, because the inoculum will be too heavy.

2. Incubate at 35°-37°C for up to 7 days.
3. Observe for growth and the development of blue colour, denoting alkalinization.

Expected Results

Positive: Growth on the medium, with or without a change in the colour of the indicator. Growth typically results in the bromthymol blue indicator turning from green to blue.

Citric acid → Oxaloacetic acid + Acetic acid→Excess in Na ions +Excess of CO_2 +Pyruvic acid

Negative: Absence of growth.

6 - Motility test: stab method in semi-solid media.

Purpose

These tests are used to determine whether an enteric organism is motile. An organism must have flagella to be motile.

Principle

The inoculum is stabbed into the centre of a semisolid agar deep. Bacterial motility is evident by a diffuse zone of growth extending out from the line of inoculation. Some organisms grow throughout the entire medium, whereas others show small areas or nodules that grow out from the line of inoculation.

Media

Enzymatic digest of gelatin (10 g), beef extract (3 g), NaCl (5 g), agar (4 g), per 1000 mL, pH 7.3.

Method

1. Touch a straight needle to a colony of a young (18- to 24-hour) culture growing on agar medium.

2. Stab once to a depth of only ⅓ to ½ inch in the middle of the tube.
3. Incubate at 35°-37°C and examine daily for up to 7 days.

Expected Results

Positive: Motile organisms will spread out into the medium from the site of inoculation.

Negative: Nonmotile organisms remain at the site of inoculation.

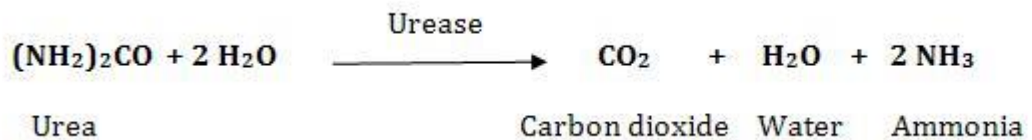
7- Urease test

Purpose

This test is used to determine an organism's ability to produce the enzyme urease, which hydrolyses urea. Proteus sp. may be presumptively identified by the ability to rapidly hydrolyse urea.

Principle

Urea is the product of decarboxylation of amino acids. Hydrolysis of urea produces ammonia and CO_2. The formation of ammonia alkalinizes the medium, and the pH shift is detected by the colour change of phenol red from light orange at pH 6.8 to magenta (pink) at pH 8.1. Rapid urease-positive organisms turn the entire medium pink within 24 hours. Weakly positive organisms may take several days, and negative organisms produce no colour change or yellow as a result of acid production.



Media: Enzymatic digest of gelatin (1 g), dextrose (1 g), NaCl (5 g), KH2PO4 (2 g), urea (20 g), phenol red (0.012 g), per 1000 mL, pH6.8.

Method

1. Streak the surface of a urea agar slant with a portion of a well-isolated colony or inoculate slant with 1 to 2 drops from an overnight brain-heart infusion broth culture.
2. Leave the cap on loosely and incubate the tube at 35°-37°C in ambient air for 48 hours to 7 days.

Expected Results

Positive: Change in colour of slant from light orange to magenta.

Negative: No colour change (agar slant and butt remain light orange).

Limitations

Alkaline reactions may appear after prolonged incubation and may be the result of peptone or other protein utilization raising the pH. To eliminate false-positive reactions, perform a control test with the base medium without urea.

Lab 3

Enterobacteriaceae: *Proteus*

Lab diagnostic tests:

1. Gram stain: Gram negative bacilli or coccobacilli or pleomorphic.
2. Inoculation MacConkey agar gives pale colonies (LNF).
3. Blood agar (Swarming and hemolysis).
4. TSI.
5. Urease test.
6. IMViC.
7. Gelatinee liquefication.
8. Phenylalanine deaminase.
9. Ornithine decarboxylase.
10. Maltose fermentation
11. Glucose fermentation

1- Gelatine liquefaction

Purpose

The production of gelatinases capable of hydrolyzing gelatine is used as a presumptive test for the identification of various organisms, including *Staphylococcus* sp., Enterobacteriaceae, and some gram-positive bacilli.

Principle

This test is used to determine the ability of an organism to produce extracellular proteolytic enzymes (gelatinases) that liquefy gelatine, a component of vertebrate connective tissue. Nutrient gelatine medium differs from traditional microbiology media in that the solidifying agent (agar) is replaced with gelatine. When an organism produces gelatinase, the enzyme liquefies the growth medium.

Media: Enzymatic digest of gelatine (5 g), beef extract (3 g), gelatine (120 g), per 1000 mL, pH 6.8.

Method

1. Inoculate the gelatine deep with 4 to 5 drops of a 24-hour broth culture.
2. Incubate at 35°-37°C in ambient air for up to 14 days. Note: Incubate the medium at °25C if the organism grows better at 25°C than at 35°C.
3. Alternatively, inoculate the gelatine deep from a 24-hour-old colony by stabbing four or five times, 0.5 inch into the medium.
4. Remove the gelatine tube daily from the incubator and place at 4°C to check for liquefaction. Do not invert or tip the tube, because sometimes the only discernible liquefaction occurs at the top of the deep where inoculation occurred.
5. Refrigerate an uninoculated control along with the inoculated tube. Liquefaction is determined only after the control has hardened (gelled).

Expected Results

Positive: Partial or total liquefaction of the inoculated tube (the control tube must be completely solidified) at 4°C within 14 days.

Negative: Complete solidification of the tube at °4C.

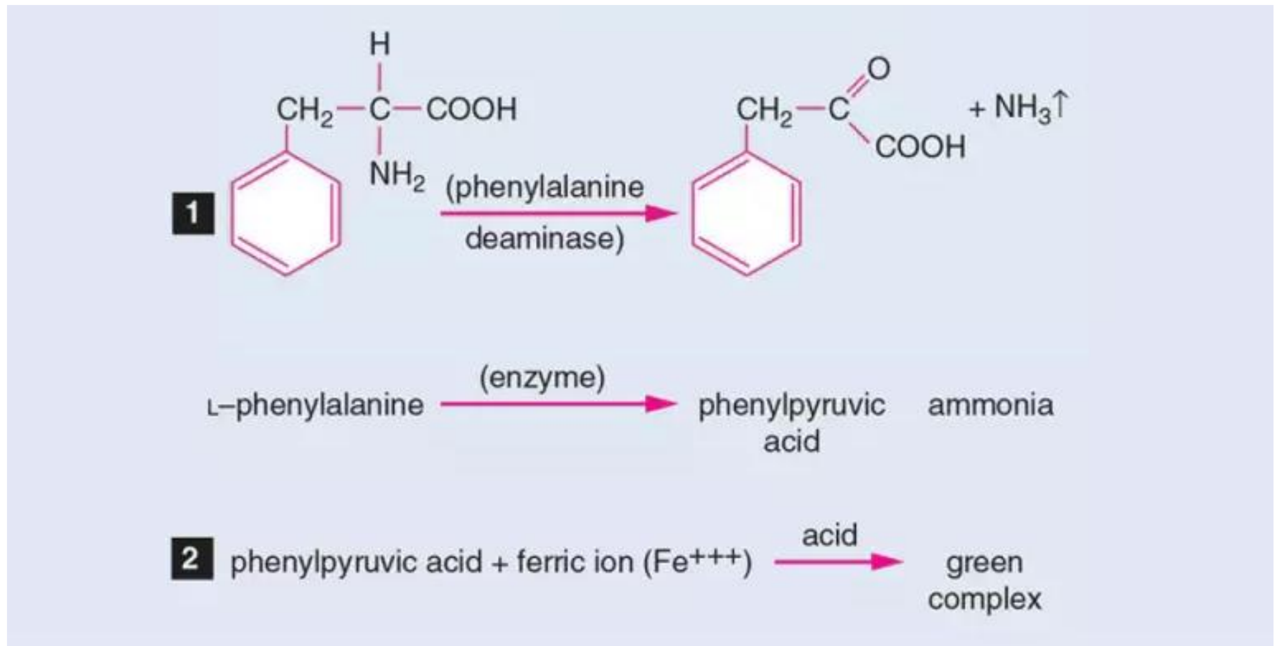
2- Phenylalanine deaminase

Purpose

This test is used to determine the ability of an organism to oxidatively deaminate phenylalanine to phenylpyruvic acid.

Principle

Microorganisms that produce phenylalanine deaminase remove the amine (NH_2) from phenylalanine. The reaction results in the production of ammonia (NH_3) and phenylpyruvic acid. The phenylpyruvic acid is detected by adding a few drops of 10% ferric chloride; a green coloured complex is formed between these two compounds.



Media

Phenylalanine (2 g), yeast extract (3 g), NaCl (5 g), Na3PO4 (1 g), agar (12 g), per 1000 mL, pH 7.3.

Method

1. Inoculate phenylalanine slant with 1 drop of a 24-hour brain-heart infusion broth.
2. Incubate 18 to 24 hours (or until good growth is apparent) at 35°-37°C in ambient air with cap loose.
3. After incubation, add 4 to 5 drops of 10% aqueous ferric chloride to the slant.

Expected Results

Positive: Green colour develops on slant after ferric chloride is added.

Negative: Slant remains original colour after the addition of ferric chloride.

3- Ornithine decarboxylase

Purpose

This test is used to differentiate decarboxylase-producing Enterobacteriaceae from other gram-negative rods.

Principle

This test measures the enzymatic ability (decarboxylase) of an organism to decarboxylate (or hydrolyse) an amino acid to form an amine. Decarboxylation, or hydrolysis, of the amino acid results in an alkaline pH and a colour change from orange to purple.

Media:

Peptic digest of animal tissue (5 g), beef extract (5 g), bromcresol purple (0.1 g), cresol red (0.005 g), dextrose (0.5 g), pyridoxal (0.005 g), ornithine (10 g), pH 6.0.

Method

A. Glucose-Nonfermenting Organisms

1. Prepare a suspension (≥McFarland No. 5 turbidity standard) in brain-heart infusion broth from an overnight culture (18 to 24 hours old) growing on 5% sheep blood agar.
2. Inoculate each of the three decarboxylase broths (arginine, lysine, and ornithine) and the control broth (no amino acid) with 4 drops of broth.
3. Add a 4-mm layer of sterile mineral oil to each tube.
4. Incubate the cultures at 35°-37°C in ambient air. Examine the tubes at 24, 48, 72, and 96 hours.

B. Glucose-Fermenting Organisms

1. Inoculate tubes with 1 drop of an 18- to 24-hour brain-heart infusion broth culture.

2. Add a 4-mm layer of sterile mineral oil to each tube.
3. Incubate the cultures for 4 days at 35°-37°C in ambient air. Examine the tubes at 24, 48, 72, and 96 hours.

Expected Results

Positive: Alkaline (purple) colour change compared with the control tube.

Negative: No colour change or acid (yellow) colour in test and control tube. Growth in the control tube.

4- Carbohydrate (Sugar) fermentation test

Purpose

Fermentation media are used to differentiate organisms based on their ability to ferment carbohydrates incorporated into the basal medium.

Principle

Carbohydrate fermentation is the process microorganisms use to produce energy. Most microorganisms convert glucose to pyruvate during glycolysis; however, some organisms use alternate pathways. A fermentation medium consists of a basal medium containing a single carbohydrate (glucose, lactose, or sucrose) for fermentation. However, the medium may contain various colour indicators, such as Andrade's indicator, bromocresol, or others. In addition to a colour indicator to detect the production of acid from fermentation, a Durham tube is placed in each tube to capture gas produced by metabolism.

Basal media:

Pancreatic digest of casein (10 g), beef extract (3 g), NaCl (5 g), carbohydrate (10 g), specific indicator (Andrade's indicator [10 mL, pH 7.4] or bromocresol purple [0.02 g, pH 6.8]).

Method

Peptone Medium with Andrade's Indicator (for Enterics and Coryneforms)

1. Inoculate each tube with 1 drop of an 18- to 24-hour brain-heart infusion broth culture.
2. Incubate at 35°-37°C for up to 7 days in ambient air.
   Note: Tubes are held only 4 days for organisms belonging to the Enterobacteriaceae family.
3. Examine the tubes for acid (indicated by a pink colour) and gas production.
4. Tubes must show growth for the test to be valid. If no growth in the fermentation tubes or control is seen after 24 hours of incubation, add 1 to 2 drops of sterile rabbit serum per 5 mL of fermentation broth to each tube.

Expected Results

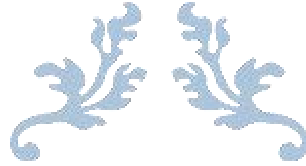
Positive: Indicator change to pink with or without gas formation in Durham tube.

Negative: Growth, but no change in colour. Medium remains clear to straw coloured.

Limitations

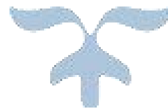
Readings after 24 hours may not be reliable if no acid is produced. No colour change or a result indicating alkalinity may occur if the organism deaminates the peptone, masking the evidence of carbohydrate fermentation.

Test	*P. mirabilis*	*P. vulgaris*	*Morganella*	*Providencia*
Urease	+	+		
TSI (H_2S)	+	+	-	-
MacConkey agar	L.N.F	L.N.F		
Indole	-	+		
MR	+	+		
VP	V	-		
Citrate	+ (v)	(v)		
Gelatine liquefaction	+	+	-	-
Phenylalanine deaminase	+	+		
Ornithine decarboxylase	-	+	+	-
Maltose	-	+		
Glucose (gas)	+	+	+	-
Sucrose	+	-	-	-
Motility	+	+		
Blood agar	Swarming + Hemolysis	Swarming + Hemolysis		



PSEUDOMONAS

Lab four



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DEPARTMENT OF BIOLOGY

COLLEGE OF SCIENCE

University of Baghdad

Lab 4

Family: Pseudomonadaceae

Genus: *Pseudomonas*

Lab diagnosis tests:

1. **Smear of Gram stain.**
2. **Oxidase test**

Purpose

This test determines the presence of cytochrome oxidase activity in microorganisms for the identification of oxidase-negative Enterobacteriaceae, differentiating them from other gram-negative bacilli.

Principle

To determine the presence of bacterial cytochrome oxidase using the oxidation of the substrate tetramethyl-p-phenylenediamine dihydrochloride to indophenol, a dark purple-coloured end product. A positive test (presence of oxidase) is indicated by the development of a dark purple colour. No colour development indicates a negative test and the absence of the enzyme.

Method

1. Moisten filter paper with the substrate (1% tetramethyl-p-phenylenediamine dihydrochloride) or select a commercially available paper disk that has been impregnated with the substrate.
2. Use a platinum wire or wooden stick to remove a small portion of a bacterial colony (preferably not more than 24 hours old) from the agar surface and rub the sample on the filter paper or commercial disk.

3. Observe the inoculated area of paper or disk for a colour change to deep blue or purple within 10 seconds (timing is critical).

Expected Results

Positive: Development of a dark purple colour within 10 seconds.

Negative: Absence of colour.

Limitations

Using nickel-base alloy wires containing chromium and iron (nichrome) to rub the colony paste onto the filter paper may cause false-positive results.

3- Culture on:

A. Milk agar for pigmentation.
B. Cetrimide agar

Purpose

This test is primarily used to isolate and purify *P. aeruginosa* from contaminated specimens.

Principle

The test is used to determine the ability of an organism to grow in the presence of cetrimide, a toxic substance that inhibits the growth of many bacteria by causing the release of nitrogen and phosphorous, which slows or kills the organism. *P. aeruginosa* is resistant to cetrimide.

Media: Enzymatic digest of gelatine (20 g), $MgCl_2$ (1.4 g), K_2SO_4 (10 g), cetrimide (cetyltrimethylammonium bromide) (0.3 g), agar (13.6), pH 7.2.

Method

1. Inoculate a cetrimide agar slant with 1 drop of an 18- to 24-hour brain-heart infusion broth culture.
2. Incubate at 35°-37°C for up to 7 days.
3. Examine the slant for bacterial growth.

Expected Results

Positive: Growth, variation in colour of colonies. *P.* aeruginosa develops yellow-green to blue-green colonies

Negative: No growth.

Limitations

Some enteric organisms will grow and exhibit a weak yellow colour in the media. This colour change is distinguishable from the production of fluorescein.

C. Blood agar.

D. King A (for pyocyanin production)

KING A MEDIUM (*Pseudomonas* P Agar) is prepared for the detection and differentiation of *P. aeruginosa* from other *Pseudomonas* based on pyocyanin production and fluorescein (pyoverdin) inhibition.

This medium contains Gelatine pancreatic digest as a rich nitrogen source, and other nutrients for growth as vitamins, minerals and amino acids. Gelatine peptone is low in phosphorous to reduce the inhibitory action on pyocyanin production. Potassium sulphate and Magnesium chloride provide cations to activate pyocyanin production and enhance pigment

production. Glycerol is a carbon source. Bacteriological agar is the solidifying agent.

Inoculate and incubate at 35 ± 2°C for 18 - 24 hours.

This medium promotes the production of pyocyanin, a blue-green pigment which oxidizes to brown, is water-soluble and, unlike fluorescein, is soluble in chloroform. The pigment diffuses throughout the medium and the blue colour is observed. Confirmation of pyocyanin production is by chloroform extraction. Add 2 ml of chloroform to a tube of medium and shake gently to remove pigment. *P. aeruginosa* gives blue colour colonies.

E. King B (for fluorescein production)

Principle and Interpretation: King Agar B enhances the elaboration of fluorescein and inhibits the pyocyanin formation. Mixed peptone provides the essential nitrogenous nutrients, carbon, sulphur and trace elements. Glycerol serves as a C-source and Dipotassium hydrogen phosphate buffers the medium. Magnesium sulphate is necessary for the activation of fluorescein production. Most pyocyanin-producing *Pseudomonas* strain synthesize also fluorescein and others produce just one pigments. The temperature can be a determining factor as most fluorescent strains will not grow at 35°C. Rather, they grow 25-35°C.

F. MacConkey agar.

G. TSI

3. **IMViC.**
4. **Motility.**
5. **Growth at 42°C.**

Purpose

This test is used to differentiate a pyocyanogenic pseudomonads from other *Pseudomonas* spp.

Principle

The test is used to determine the ability of an organism to grow at 42°C. Several *Pseudomonas* species have been isolated in the clinical laboratory that are capable of growth at elevated temperatures.

Method

1. Inoculate two tubes of trypticase soy agar (TSA) with a light inoculum by lightly touching a needle to the top of a single 13- to 24-hour-old colony and streaking the slant.
2. Immediately incubate one tube at 35°C and one at 42°C.
3. Record the presence of growth on each slant after 18 to 24 hours.

Expected Results

Positive: Good growth at both 35°and 42°C (ex. *P. aeruginosa*).

Negative: No growth at 42°C, but good growth at 35°C (ex. *P. fluorescens*).

6. **OF (Oxidation Fermentation)**.

Purpose

This test is used to differentiate microorganisms based on the ability to oxidize or ferment specific carbohydrates.

Principle

This test is used to determine whether an organism uses carbohydrate substrates to produce acid byproducts. Non-fermentative bacteria are routinely tested for their ability to produce acid from six carbohydrates (glucose, xylose, mannitol, lactose,

sucrose, and maltose). In addition to the six tubes containing carbohydrates, a control tube containing the OF base without carbohydrate is also inoculated. Triple sugar iron agar (TSI) is also used to determine whether an organism can ferment glucose. OF glucose is used to determine whether an organism ferments (Figure 2, A) or oxidizes (Figure 2, B) glucose. If no reaction occurs in either the TSI or OF glucose, the organism is considered a non-glucose utilizer (Figure 2, C). Two tubes are required for interpretation of the OF test. Both are inoculated, and one tube is overlaid with mineral oil, producing an anaerobic environment. Production of acid in the overlaid tube results in a colour change and is an indication of fermentation. Acid production in the open tube and colour change is the result of oxidation.

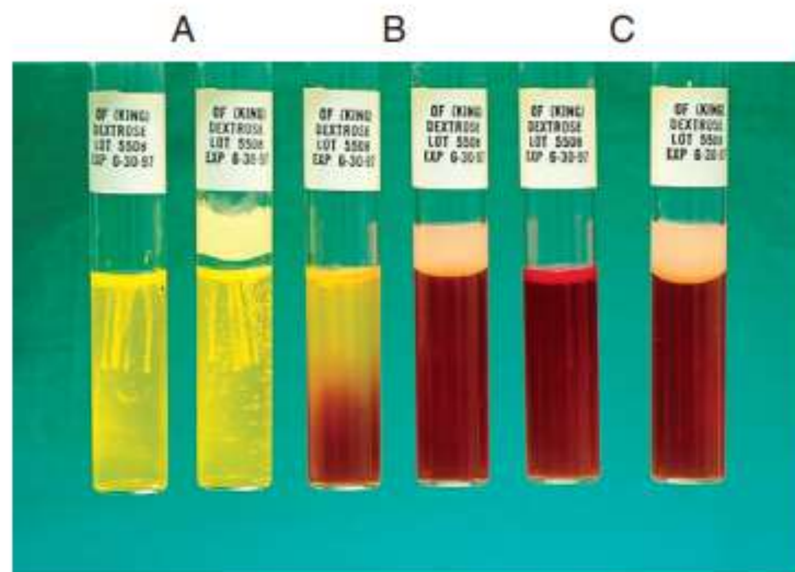


Figure 2 Oxidation/fermentation medium (CDC method). **A,** Fermenter. **B,** Oxidizer. **C,** Nonutilizer.

Media: Pancreatic digest of casein (2 g), glycerol (10.0 mL), phenol red (King method) (0.03 g), agar (3 g), per 1000 mL, pH 7.3.

Method

1. To determine whether acid is produced from carbohydrates, inoculate agar deeps, each containing a single carbohydrate, with bacterial growth from an 18- to 24-hour culture by stabbing a needle 4 to 5 times into the medium to a depth of 1 cm. Note: Two tubes of OF dextrose are usually inoculated; one is overlaid with either sterile melted petrolatum or sterile paraffin oil to detect fermentation.
2. Incubate the tubes at 35°-37°C in ambient air for up to 7 days. Note: If screwcap tubes are used, loosen the caps during incubation to allow for air exchange. Otherwise, the control tube and tubes containing carbohydrates that are not oxidized might not become alkaline.

Expected Results

Positive: Acid production (A) is indicated by the colour indicator changing to yellow in the carbohydrate-containing deep.

Weak-positive (Aw): Weak acid formation can be detected by comparing the tube containing the medium with carbohydrate with the inoculated tube containing medium with no carbohydrate. Most bacteria that can grow in the OF base produce an alkaline reaction in the control tube. If the colour of the medium in a tube containing carbohydrate remains about the same as it was before the medium was inoculated and if the inoculated medium in the control tube becomes a deeper red (i.e., becomes alkaline), the culture being tested is considered weakly positive, assuming the amount of growth is about the same in both tubes.

Negative: Red or alkaline (K) colour in the deep with carbohydrate equal to the colour of the inoculated control tube.

No change (NC) or neutral (N): There is growth in the media, but neither the carbohydrate containing medium nor the control base turns alkaline (red).

Note: If the organism does not grow at all in the OF medium, mark the reaction as no growth (NG).

Limitations

Slow-growing organisms may not produce results for several days.

8- Nitrate reduction test.

Purpose

This test is used to determine the ability of an organism to reduce nitrate to nitrite. All members of the Enterobacteriaceae family reduce nitrate, but some members further metabolize nitrite to other compounds.

Principle

Anaerobic metabolism requires an electron acceptor other than atmospheric oxygen (O_2). Many gram-negative bacteria use nitrate as the final electron acceptor. The organisms produce nitrate reductase, which converts the nitrate (NO_3) to nitrite (NO_2). The reduction of nitrate to nitrite is determined by adding sulfanilic acid and alpha-naphthylamine. The sulfanilic acid and nitrite react to form a diazonium salt. The diazonium salt then couples with the alpha-naphthylamine to produce a red, water-soluble azo dye. If no colour change occurs, the organism did not reduce nitrate or reduced it further to NH_3, NO, or N_2O_2. Zinc is added at this point; if nitrate remains, the zinc will reduce the compound to nitrite and the reaction will turn positive, indicating a negative test result for nitrate

reduction by the organism. If no colour change occurs after the addition of zinc, this indicates that the organism reduced nitrate to one of the other nitrogen compounds previously described.

A Durham tube is placed in the broth for two reasons: (1) to detect deterioration of the broth before inoculation, as evidenced by gas formation in the tube; and (2) to identify denitrification by organisms that produce gas by alternate pathways; if gas is formed in the tube before the addition of the colour indicator, the test result is negative for nitrate reduction by this method.

Media: Pancreatic digest of gelatine (20 g), KNO_3 (2 g), per 1000 mL.

Method

1. Inoculate nitrate broth with 1 to 2 drops from a young broth culture of the test organism.
2. Incubate for 48 hours at 35°-37°C in ambient air (some organisms may require longer incubation for adequate growth).
3. Test these cultures 24 hours after obvious growth is detected or after a maximum of 7 days.
4. After a suitable incubation period, test the nitrate broth culture for the presence of gas, reduction of nitrate, and reduction of nitrite according to the following steps:
    a. Observe the inverted Durham tube for the presence of gas, indicated by bubbles inside the tube.
    b. Add 5 drops each of nitrate reagent solution A (sulfanilic acid) and B (alpha-naphthylamine). Observe for at least 3 minutes for a red colour to develop.
    c. If no colour develops, test further with zinc powder. Dip a wooden applicator stick into zinc powder and transfer only

the amount that adheres to the stick to the nitrate broth culture to which solutions A and B have been added. Observe for at least 3 minutes for a red colour to develop. Breaking the stick into the tube after the addition of the zinc provides a useful marker for the stage of testing.

Expected Results

The nitrate reduction test is read for the presence or absence of three metabolic products: gas, nitrate (NO_3), and nitrite (NO_2). The expected results can be summarized as follows:

Reaction	Gas	Colour after Addition of Solutions A and B	Colour after Addition of Zinc	Interpretation
$NO_3 \rightarrow NO_2$ (Figure 1, A)	None	Red	—	NO_3+, no gas
$NO_3 \rightarrow NO_2$, gas partial nongaseous end products	None	Red	—	NO_3+, no
$NO_3 \rightarrow NO_2$, gaseous end products (Figure 1, B)	Yes	Red	—	NO_3+, gas+
$NO_3 \rightarrow$ gaseous end product (Figure 1, C)	Yes	None	None	NO_3+, NO_2+, gas+ C
$NO_3 \rightarrow$ nongaseous end products	None	None	None	NO_3+, NO_2+, no gas
$NO_3 \rightarrow$ no reaction	None	None	Red	Negative

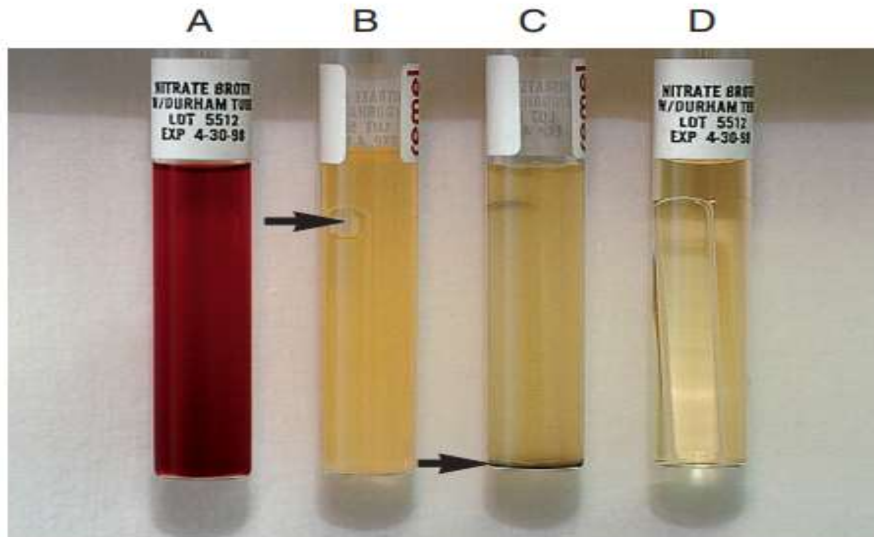


Figure 1 Nitrate reduction. **A,** Positive, no gas. **B,** Positive, gas *(arrow)*. **C,** Positive, no color after addition of zinc *(arrow)*. **D,** Uninoculated tube.

Test	*P. aeruginosa*	*P. fluorescens*
Indole	-	-
MR	-	-
VP	-	-
SC	+	+
TSI	K/K - -	K/K - -
Nitrate	+	+
Motility	+	+
Growth at 42	+	-
Growth at 4 C	-	+
King A	+ pyocyanin	- Pyocyanin
King B	+ fluorescein	+ fluorescein
MacConkey	L.N.F transparence, irregular	L.N.F transparence, irregular
Blood agar	Spreading and flat, serrated edges; often shows metallic sheen; bluish green, red, or brown pigmentation; colonies often beta–haemolytic; grapelike or corn tortilla–like odour; mucoid colonies	No distinctive appearance
Oxidase	+	+
Nitrate reduction	NO_3+, gas	NO_3+, gas
OF medium	Oxidation (+), ferm(-)	Oxidation (+), ferm (-)

Lab 5: *Salmonella* and *Shigella*

A- *Salmonella*

The diagnosis of salmonellosis requires bacteriologic isolation of the organisms from appropriate clinical specimens. Laboratory identification of the genus *Salmonella* is done by biochemical tests; the serologic type is confirmed by serologic testing. Faeces, blood, or other specimens should be plated on several nonselective and selective agar media (blood, MacConkey, bismuth sulphite, *Salmonella-Shigella*, CLED, XLD, and brilliant green agars) as well as enrichment broth such as selenite or tetrathionate. Any growth in enrichment broth is subsequently subcultured onto the various agars.

The biochemical reactions of suspicious colonies are then determined. The presumptive biochemical identification of *Salmonella* then can be confirmed by antigenic analysis of O and H antigens using polyvalent and specific antisera. Fortunately, approximately 95% of all clinical isolates can be identified with the available group A-E typing antisera.

1- Microscopic Appearance

Gram's stain: Gram negative rods

2- Culture Media

- **Blood agar**

  Specimens are incubated in 5-10% CO_2 at 35–37°C for 16-24hrs. Colonies are moist and 2-3mm in diameter.

- **MacConkey agar**

  Typical colonies appear transparent and colourless, sometimes with dark centre. Colonies of Salmonella will clear areas of precipitated bile caused by other organisms sometimes present.

- **Cystine-lactose electrolyte-deficient (CLED) agar:**
Specimens are incubated in air at 35–37°C for 16-24hrs. *Salmonella* species are lactose non fermenters (some serotypes e.g. *S.* Arizonae and *S.* Indiana may ferment lactose). Enteric bacteria on CLED agar. Bacteria who utilize lactose form yellow colonies on CLED (typically *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*). Bacteria who don't utilize lactose (typically *Salmonella* spp., *Shigella*, *Proteus* spp.) form blue-green colonies on the surface of CLED agar. The lack of electrolytes suppresses the swarming of *Proteus*.

- **Xylose-lysine desoxycholate (XLD) agar**
XLD Agar is both a selective and differential medium. This medium inhibit growth of most Enterobacteriaceae, allowing *Salmonella* and *Shigella* spp. to be detected. incubated in air at 35–37°C for 16-24hrs. Colonies are red, and usually with a black centre. Shigella develop red colonies.

- **Bismuth Sulphite Agar**
Peptone and HM Peptone B serve as sources as carbon, nitrogen, long chain amino acids, vitamins, and essential growth factors. Dextrose is the carbon source. Disodium phosphate maintains the osmotic equilibrium. Bismuth sulphite indicator along with brilliant green inhibits the intestinal gram-positive and gram-negative bacteria. Ferrous sulphate aids in the detection of hydrogen sulphide production. *Salmonella enterica* serotype Typhi, *Salmonella enterica* serotype Enteritidis, and *Salmonella enterica* serotype Typhimurium typically grow as black colonies with a surrounding metallic sheen resulting from hydrogen sulphide production and

reduction of sulphite to black ferric sulphide. *S*. Paratyphi A grows as light green colonies.

- ***Salmonella Shigella* agar**

  Salmonella Shigella agar is a moderately selective medium in which gram-positive bacteria are inhibited and it is highly selective for *Salmonella* species, but is inhibitory to some strains of *Shigella*. The inclusion of Bile Salts, Sodium Citrate and Brilliant Green serve to inhibit gram-positive, coliform organisms and inhibit swarming *Proteus* spp., while allowing *Salmonella* spp. to grow. Lactose is the carbohydrate present in *Salmonella Shigella* Agar. Thiosulfate and Ferric Citrate permit detection of hydrogen sulphide by the production of colonies with black centres. Neutral red turns red in the presence of an acidic pH, thus showing fermentation has occurred.

3- Biochemical tests

- Oxidase (negative)
- Urease (negative)
- IMViC (negative, positive, negative, negative)
- TSI (K/A + +)
- Nitrate reduction (positive)
- Glucose fermentation (positive)

4- Motility test

5- Agglutination test

Test	*S.* Typhi	*S.* Typhimurium
Gram stain	Gram-negative rods	Gram-negative rods
Blood agar	Colonies are moist and 2-3mm in diameter	Colonies are moist and 2-3mm in diameter
MacConkey	L. N. F.	L. N. F.
XLD	blue-green colonies	blue-green colonies
CLED	yellow-orange or pink coloured colonies	Colonies are red, and usually with a black centre
BSA	black colonies with a surrounding metallic sheen	black colonies with a surrounding metallic sheen
SS agar	Colourless colonies	Colourless colonies with black centre
Urease	-	-
Oxidase	-	-
Nitrate reduction	+	+
Indole	-	-
Methyl red	+	+
VP	-	-
Citrate utilization	-	+
TSI	K/A, (+), -	K/A, +, +
Glucose fermentation	+	+
Motility	+	+

B- *Shigella*

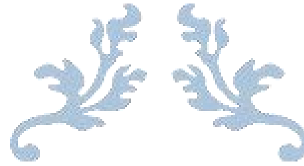
It includes four species; *Shigella flexneri*, *Shigella dysenteriae*, *Shigella boydii,* and *Shigella sonnei.* In case of dysentery, fresh stool is preferable for direct diagnosis. WBC & RBC may be seen. Thereafter, stool should be cultured on appropriate media.

Lab diagnosis

It involves several morphological and biochemical test:

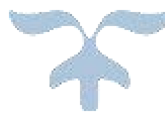
1- Gram stain.
2- Culture on:
    - MacConkey agar.
    - XLD.
    - CLED
    - S.S agar.
3- Urease.
4- IMViC.
5- Motility.
6- Glucose fermentation.
7- Mannitol fermentation.
8- TSI.

Test	*S. dysenteriae*	*S. flexneri*	*S. boydii*	*S. sonnei*
Gram stain	Gram-negative rods	Gram-negative rods	Gram-negative rods	Gram-negative rods
MacConkey agar	L. N. F.	L. N. F.	L. N. F.	L. N. F.
XLD	red colonies	red colonies	red colonies	red colonies
CLED	blue-green colonies	blue-green colonies	blue-green colonies	blue-green colonies
SS agar	Colourless colonies	Colourless colonies	Colourless colonies	Colourless colonies
TSI	K/A, -, -	K/A, -, -	K/A, -, -	K/A, -, -
Motility	-	-	-	-
Mannitol	-	+	+	+
Ornithine decarboxylase	-	-	-	+
Urease	-	-	-	-
IMViC	V, +, -, -	V, +, -, -	V, +, -, -	V, +, -, -



STAPHYLOCOCCUS

Lab six



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Lab 6: *Staphylococcus*

Lab diagnostic tests:

1. **Gram stain.**
2. **Culture on:**
    a. Blood agar (haemolysis).
    b. Milk agar (pigmentation).
    c. Mannitol salt agar (selective & differential), selective for *Staphylococcus* because it contains 7.5% NaCl, differential for *S. aureus* appear as yellow colonies due to mannitol fermentation (the indicator is phenol red).
    d. Staph 110 (selective, differential, and gelatinase activity), selective for *Staphylococcus* because it contains 7.5% NaCl. The differentiation ability is based on mannitol fermentation; which is detected by adding a few drops of bromothymol blue to the plate and looking for a yellow halo around the colonies.
3. **Biochemical tests**
    a. Oxidase
    b. Catalase

    Purpose

    This test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate those bacteria that produces an enzyme catalase, such as *staphylococci*, from non-catalase producing bacteria such as *streptococci*. Normally 3% H_2O_2 is used for the routine culture while 15% H_2O_2 is used for the detection of catalase in anaerobes. While 30% H_2O_2 is used for detecting *Neisseria*.

Principle

- The metabolic activity of aerobic and facultative anaerobic microorganisms produces toxic by-products like hydrogen peroxide and superoxide radical (O_2^{-}).
- These products are toxic to the organisms and might even result in cell lysis if not broken down. In the case of pathogenic organisms, different mechanisms are found that break down these products to non-toxic substances.
- Bacteria capable of synthesizing the enzyme catalase hydrolyse hydrogen peroxide into water and gaseous oxygen, which results in the liberation of gas bubbles.

$$2H_2O_2 \xrightarrow[\text{catalase}]{} 2H_2O + O_2$$

- The production of catalase thus protects the organism against the lethal effect of hydrogen peroxide accumulated at the end of the aerobic metabolism.

Procedure

A. Slide Method

- A microscope slide is placed inside a petri dish. The use of a petri dish is optional and is used to limit catalase aerosols, which might carry viable bacterial cells.
- A small amount of organism is collected from a well-isolated 18- to 24-hour colony with a sterile inoculating loop or wooden applicator stick and placed onto the microscope slide.

- However, no agar must be picked up with the colony, especially when the culture is picked up from blood agar.
- A drop of 3% H_2O_2 onto the organism on the microscope slide by using a dropper or Pasteur pipette.
- The formation of bubbles is observed against a dark background to enhance readability.

B. Tube Method

- About 4 to 5 drops of 3% H_2O_2 are added to a test tube.
- Using a wooden applicator stick, a small amount of organisms from a well-isolated 18- to 24-hour colony is collected and placed into the test tube.
- The tube is placed against a dark background and observed for immediate bubbles.

Limitations

- RBCs contain catalase, and thus, in order to avoid false-positive results, blood agar should not be picked up with the colony.
- The test should not be tested from Mueller-Hinton agar.
- Collecting colonies with metal bacteriological loop materials might yield false-positive results; however, platinum loops do not yield false-positive results.
- Because the enzyme is present in viable cells only, colonies that are older than 24 hours should not be used. Older cultures may give false-negative results.
- Reversing the order of adding the reagent to the colony might result in false-negative results.
- The reagent and the colony should not be mixed.

- Some strains of *S. aureus* may appear catalase-negative by drop method so the test should be repeated with the tube method.

c. Acetoin production

As in Voges-Proskauer test

d. Coagulase test

Purpose

Coagulase test is used to differentiate *Staphylococcus* aureus (positive) which produce the enzyme coagulase, from *S. epidermis* and *S. saprophyticus* (negative) which do not produce coagulase. i.e Coagulase Negative staphylococci (CONS).

Principle

Coagulase is an enzyme-like protein and causes plasma to clot by converting fibrinogen to fibrin. coagulase involves the activation of plasma coagulase-reacting factor (CRP), which is a modified or derived thrombin molecule, to from a coagulase-CRP complex. This complex in turn reacts with fibrinogen to produce the fibrin clot.

Procedure

1. Add 0.5 ml of broth or few colonies to 0.5 ml citrated or oxalated human or rabbit plasma after dilution to 1:5.
2. Incubate at 35-37°C. If clots form within 1 – 4 hr, the test is positive. Otherwise, the test is negative.

e. Clumping factor

Principle

Previously, it was known as bound coagulase. Clumping factor is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in an alternation of fibrinogen so that it precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma. This doesn't require coagulase-reacting factor.

Procedure

1. Place a drop of physiological saline on each end of a slide, or on two separate slides.
2. With the loop, straight wire or wooden stick, emulsify a portion of the isolated colony in each drop to make two thick suspensions.
3. Add a drop of human or rabbit plasma to one of the suspensions, and mix gently.
4. Look for clumping of the organisms within 10 seconds.
5. No plasma is added to the second suspension to differentiate any granular appearance of the organism from true coagulase clumping.

f. DNase

Purpose

- To determine the ability of an organism to produce the DNase enzyme.

- To differentiate and identify *S. aureus* from other Staphylococcal species.

Principle

- The deoxyribonuclease enzyme produced by bacteria is extracellular endonucleases that break down DNA, yielding a high concentration of oligonucleotides.
- The media used to detect these enzymes can be made by using various indicators (toluidine blue or methyl green) or no indicators to detect the hydrolysis of DNA.
- The first method is performed with no indicator. The hydrolysis of DNA is indicated by the clearing of the agar after the addition of HCl (the oligonucleotides dissolve in acid causing a clear zone, but DNA salts are insoluble).
- When methyl green indicator is added, DNA combines with the methyl green to produce a green colour.
- The complex is released when the DNA is hydrolysed, and the freed methyl green is colourless at pH 7.5.
- When toluidine blue O (TBO) is added, a complex is formed with the DNA, which changes its structure when DNA is hydrolysed, resulting in a bright pink colour.

Procedure

Media

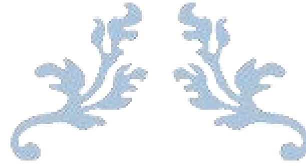
- DNase Test Agar Base is used for testing the production of the DNase enzyme. The medium can also be added with indicators like methyl green or toluidine blue O.
- The composition of the DNase Test Agar Base is tryptone (15 g/l), soya peptone (5 g/l), DNA (2 g/l), NaCl (5 g/l), and (15 g/l) agar. Final pH at 25°C: 7.3 ±0.2

Protocol

1. **Without indicator**
   - The agar plates are inoculated with the test organism from an 18-hour culture with a sterile inoculating loop or needle.
   - The plates are incubated at 35-37°C for 24 hours.
   - The incubated agar plates are flooded with a 1N HCl solution, and the excess acid is tipped off.
   - Some time is allowed for the reagent to be absorbed into the plates.
   - The plates are observed for a clear zone around the colonies within 5 minutes.

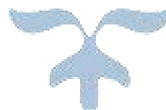
2. **With indicator**
   - The agar plates with an indicator are inoculated with the test organism from an 18-hour culture with a sterile inoculating loop or needle.
   - The plates are incubated for 24 hours at 35-37°C.
   - The plates are then observed for the change in colour of the indicator.

Test	*S. aureus*	*S. epidermidis*	*S. saprophyticus*
Gram stain	Gram positive cocci	Gram positive cocci	Gram positive cocci
Blood agar (haemolysis)	β-haemolysis -haemolysisα	γ-haemolysis	γ-haemolysis
Pigment on Milk agar	Golden	White	Light yellow
Mannitol salt agar/Growth	+	+	+
Mannitol salt agar/fermentation	Yellow	Red	Red
Staph 110	+	+	+
Oxidase	-	-	-
Catalase	+	+	+
Acetoin production	+	-	-
Coagulase	+	-	-
Clumping factor	+	-	-
DNase	+	-	-



STREPTOCOCCI

Lab seven



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Lab 6: Streptococcaceae

Lab diagnostic tests:

1. **Gram stain.**
2. **Catalase.**
3. **Bile solubility**

Purpose

This test differentiates *Streptococcus pneumoniae* (positive–soluble) from α-hemolytic streptococci (negative–insoluble).

Principle

Bile or a solution of a bile salt (e.g., sodium desoxycholate) rapidly lyses pneumococcal colonies. Lysis depends on the presence of an intracellular autolytic enzyme, amidase. Bile salts lower the surface tension between the bacterial cell membrane and the medium, thus accelerating the organism's natural autolytic process.

Method

1. After 12 to 24 hours of incubation on 5% sheep blood agar, place 1 to 2 drops of 10% sodium desoxycholate on a well- isolated colony. Note: A tube test is performed with 2% sodium desoxycholate.
2. Gently wash liquid over the colony without dislodging the colony from the agar.
3. Incubate the plate at 35°-37°C in ambient air for 30 minutes.
4. Examine for lysis of colony.

Expected Results

Positive: Colony disintegrates; an imprint of the lysed colony may remain in the zone.

Negative: Intact colonies

4. **Blood agar (5% sheep blood)**

   It is used for detecting of the type of haemolysis. Furthermore, it also used for isolating group A streptococci from throat swabs, after supplementing with trimethoprim-sulfamethoxazole to suppress the growth of normal flora. However, this medium also inhibits growth of groups C, F, and G α-hemolytic streptococci.

5. **Streptokinase test**

   A streptococcal growth is added to plasma clot. The lysis of this clot, is an indication of streptokinase production. All *Streptococcus* species produce streptokinase except *S. pneumoniae*.

6. **Carbohydrate fermentation:**

   To differentiate *Streptococcus* spp., this test is performed. The Mueller Hinton agar with carbohydrate is used for fermentation, the indicator is bromothymol blue better than phenol red, the sugars are glucose, inulin, mannitol & lactose.

7. **Bacitracin susceptibility**

Purpose

This test is used for presumptive identification and differentiation of β-hemolytic group A streptococci from other β-hemolytic streptococci. It is also used to distinguish staphylococci species (resistant) from micrococci (susceptible).

Principle

The antibiotic bacitracin inhibits the synthesis of bacterial cell walls. A disk impregnated with a small amount of bacitracin (0.04 units) is placed on an agar plate, allowing the antibiotic to diffuse into the medium and inhibit the growth of susceptible organisms. After incubation, the

inoculated plates are examined for zones of inhibition surrounding the disks.

Method

1. Using an inoculating loop, streak two or three suspect colonies of a pure culture onto a blood agar plate.
2. Using heated forceps, place a bacitracin disk in the first quadrant (area of heaviest growth). Gently tap the disk to ensure adequate contact with the agar surface.
3. Incubate the plate for 18 to 24 hours at °37-°35C in ambient air for staphylococci and in %5 to %10 carbon dioxide (CO_2) for streptococci differentiation.
4. Look for a zone of inhibition around the disk.

Expected Results

Positive: Any zone of inhibition greater than 10 mm; susceptible.

Negative: No zone of inhibition; resistant.

8. Optochin test

Purpose

This test is used to determine the effect of Optochin (ethyl hydrocupreine hydrochloride) on an organism. Optochin lyses pneumococci (positive test), but α-streptococci are resistant (negative test).

Principle

Optochin is an antibiotic that interferes with the ATPase and production of adenosine triphosphate (ATP) in microorganisms. The Optochin- impregnated disk is placed on a lawn of organism on a sheep blood agar plate, allowing the antibiotic to diffuse into the medium. The antibiotic inhibits the growth of a susceptible organism, creating a

clearing, or zone of inhibition, around the disk. A zone of 14 to 16 mm is considered susceptible and presumptive identification for *S. pneumoniae*.

Method

1. Using an inoculating loop, streak two or three suspect colonies of a pure culture onto half of a 5% sheep blood agar plate.
2. Using heated forceps, place an Optochin disk in the upper third of the streaked area. Gently tap the disk to ensure adequate contact with the agar surface.
3. Incubate the plate for 18 to 24 hours at 35°C in 5% CO_2. Note: Cultures do not grow as well in ambient air, and larger zones of inhibition occur.
4. Measure the zone of inhibition in millimetres, including the diameter of the disk.

Expected Results

Positive: Zone of inhibition ≥ 14 mm in diameter, with 6-mm disk.

Negative: No zone of inhibition

9. Quellung reaction:

This is performed for capsulated *S. pneumoniae*, by mixing bacterial growth with standard specific anti-capsular Ag, swelling of the capsule indicates positive result.

10. CAMP reaction

Purpose

The Christie, Atkins, and Munch-Peterson (CAMP) test is used to differentiate group B streptococci (*Streptococcus agalactiae*– positive) from other streptococcal species. *Listeria monocytogenes* also produces a positive CAMP reaction.

Principle

Certain organisms (including group B streptococci) produce a diffusible extracellular haemolytic protein (CAMP factor) that acts synergistically with the beta-lysin of *S. aureus* to cause enhanced lysis of red blood cells. The group B streptococci are streaked perpendicular to a streak of *S. aureus* on sheep blood agar. A positive reaction appears as an arrowhead zone of haemolysis adjacent to the place where the two streak lines come into proximity.

Method

1. Streak a beta-lysin–producing strain of *S. aureus* down the centre of a sheep blood agar plate.
2. Streak test organisms across the plate perpendicular to the *S. aureus* streak within 2 mm. (Multiple organisms can be tested on a single plate).
3. Incubate overnight at 35-37°C in ambient air.

Expected Results

Positive: Enhanced haemolysis is indicated by an arrowhead-shaped zone of β- haemolysis at the juncture of the two organisms.

Negative: No enhancement of haemolysis.

11.Salt tolerance

Purpose

This test is used to determine the ability of an organism to grow in high concentrations of salt. It is used to differentiate enterococci (positive) from nonenterococci (negative).

Principle

The salt tolerance test is a selective and differential medium. Enterococci are resistant to high salt concentration. A heart infusion

broth containing 6.5% NaCl is used as the test medium. This broth also contains a small amount of glucose and bromocresol purple as the indicator for acid production.

Media

Brain-heart infusion broth (BHI) may be used in place of the individual components with the addition of NaCl and indicator dye. Components: Heart digest (10 g), enzymatic digest of animal tissue (10 g), NaCl (65 g), dextrose (1 g), bromocresol purple (0.016 g), per 1000 ml.

Method

1. Inoculate one or two colonies from an 18- to 24-hour culture into 6.5% NaCl broth.
2. Incubate the tube at 35°-37°C in ambient air for 48 hours.
3. Check daily for growth.

Expected Results

Positive: Visible turbidity in the broth, with or without a colour change from purple to yellow.

Negative: No turbidity and no colour change.

Test	*S. pneumoniae*	*E. faecalis*	*S. pyogenes*
Gram stain	Gram positive cocci	Gram positive cocci	Gram positive cocci
Inulin	+	-	-
Lactose	-	+	+no gas
Mannitol	-	+	+no gas
Glucose	-	+	+ no gas
Bile salt Solubility	(+) no growth	(-) growth	(-) growth
Optochin	+ no growth	(-) growth	(-) growth
Bacitracin	(-) growth	(-) growth	(+) no growth
CAMP	-	-	-
Haemolysis	α	α	B
Salt tolerance	(+) growth	(-) no growth	(-) no growth
Streptokinase	(-) clot	(+) no clot	(+) no clot

VIBRIO CHOLERAE

Lab Eight

DEPARTMENT OF BIOLOGY
COLLEGE OF SCIENCE
University of Baghdad

Lab 9: *Vibrio cholerae*

Lab diagnostic tests

A. Culture

Stool cultures for *Vibrio* spp. are plated on the selective medium thiosulfate citrate bile salts sucrose (TCBS) agar. TCBS contains 1% sodium chloride, bile salts that inhibit the growth of gram-positive organisms, and sucrose for the differentiation of the various *Vibrio* spp. Bromothymol blue and thymol blue pH indicators are added to the medium. The high pH of the medium (8.6) inhibits the growth of other intestinal flora. Although some *Vibrio* spp. grow very poorly on this medium, those that grow well produce either yellow or green colonies, depending on whether they are able to ferment sucrose (which produces yellow colonies). Alkaline peptone water (pH 8.4) may be used as an enrichment broth for obtaining growth of vibrios from stool. After inoculation, the broth is incubated for 5 to 8 hours at 35°C and then subcultured to TCBS.

B. Serological test.

C. Biochemical tests

1. **Oxidase test.**

   The oxidase test must be performed from 5% sheep blood or another medium without a fermentable sugar (e.g., lactose in MacConkey agar or sucrose in TCBS), because fermentation of a carbohydrate results in acidification of the medium, and a false-negative result may occur if the surrounding pH is below 5.1 The oxidase test must be performed from 5% sheep blood or another medium without a fermentable sugar (e.g., lactose in MacConkey agar or sucrose in TCBS), because fermentation of a carbohydrate results in acidification of the medium, and a false-negative result may occur if the surrounding pH is below 5.1

2. **String test**

   The string test may be performed on a glass microscope slide or plastic petri dish by suspending 18- to 24-hour growth from heart infusion agar (HIA) or other non-inhibitory medium in a drop of 0.5% aqueous solution of sodium deoxycholate. If the result is positive, the bacterial cells will be lysed by the sodium deoxycholate, the suspension will lose turbidity, and DNA will be released from the lysed cells causing the mixture to become viscous. A mucoid “string” is formed when an inoculating loop is drawn slowly away from the suspension. Most vibrios are positive.

3. **TSI**: A/A, no gas, and no H2S.
4. **Sugar fermentation:**

   Vibrio cholerae ferments both glucose and sucrose but does not produce gas in either carbohydrate.

5. **Ornithine decarboxylase.**

   V. cholerae is positive for ornithine decarboxylase.
6. **Salt broth:**

   The 0% and 1% salt broths (nutrient broth base) should be inoculated very lightly from fresh growth. The inoculum should be light enough to prevent visible turbidity before incubation of the broths. The broths are incubated at 35° to 37°C and read at 18 to 24 hours. In the absence of overnight growth, they may be incubated for up to 7 days. *V. cholerae* grows in the absence of NaCl, but growth is stimulated by the addition of 1% NaCl. *V. parahaemolyticus* cannot grow at 0% NaCl.

7. **VP test.**

   A modification is performed to the test medium to increase the test sensitivity with the vibrios. In this modification, the test medium (MR-VP broth) incorporates 1% NaCl, reagent A consists of 5%

alpha-naphthol in absolute ethanol, and reagent B is a solution of 0.3% creatine in 40% KOH. *V. cholerae* gives a positive reaction. The Voges-Proskauer test has been used to differentiate between the El Tor and classical biotype of V. cholerae O1. Classical biotypes usually give negative results; El Tor isolates are generally positive.

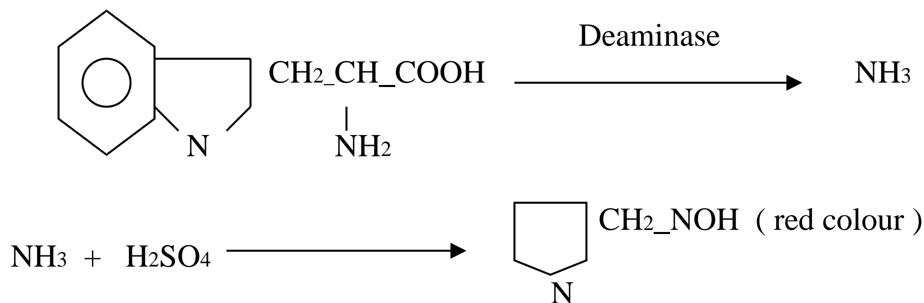
8. **Susceptibility to vibriostatic compound O/129**

   A Vibrio static test using 0/129 (2,4-diamino-6, 7-diisopropylpteridine)–impregnated disks also has been used to separate vibrios (susceptible) from other oxidase- positive glucose fermenters (resistant) and to differentiate *V. cholerae* O1 and non-O1 (susceptible) from other books *Vibrio* spp. (resistant). However, recent strains of *V. cholerae* O139 have demonstrated resistance, so the depend- ability of this test is questionable.

9. **Hemolytic activity.**
10. **Cholera red reaction.**

♣ Nitroso indol = cholera red test

Trypyophane + conc.H_2SO_4 (4 drops) ⟶ nitroso indol (red colour)



Test	*Vibrio cholerae*	*Vibrio parahaemolyticus*
Catalase	+	+
Oxidase	+	+
NO_3 reduction	+	+
Indol	+	+
MR	*+ weak*	-
VP	-	-
Citrate	+ / -	+ / -
D.W +7 % Nacl	-	+
D.W + 0 % Nacl	+	-
TSI	*A /A - -*	*K / A - -*
Motility	+	+
Cholera red	+	-
Mannitol	*+ weak*	*+ weak*
String test	+	+
OF medium	*Oxida-ferm.*	*Oxida-ferm.*
TCBS	*Yellow colonies*	*Green colonies*

BACILLUS AND CLOSTRIDIUM

Lab Nine

DEPARTMENT OF BIOLOGY
COLLEGE OF SCIENCE
University of Baghdad

Lab 9: *Bacillus* and *Clostridium*

I- *Bacillus*: *B. anthracis* and *B. cereus*

Facultative anaerobes and spore formers

Diagnostic Laboratory Tests:

1- **Gram stain**
2- **Spore stain**
3- **Culture on sheep blood agar**
4- **Catalase test**
5- **Motility test**
6- **Capsule stain**
7- **Serology**
8- **Molecular methods**

Test	***B. anthracis***	***B. cereus***
Gram stain	Greater than 1 µm in diameter	Greater than 1 µm in diameter
Spore stain	Spores do not cause swelling of cell	Spores do not cause swelling of cell
Culture on sheep blood agar	Medium-large, grey, flat, irregular with swirling projections (“Medusa head”) or ground glass appearance; nonhemolytic	Large, feathery, spreading; beta-haemolytic
Catalase	+	+
Motility	-	+
Capsule	+	-

II- *Clostridium*: *C. perfringens, C. tetani, C. botulinum*

Laboratory diagnosis:

Diagnostic Laboratory Tests:

1- **Gram stain**

Clostridium are phylogenetically heterogeneous and are Gram positive but can decolourise easily and appear Gram negative or Gram variable.

2- **Spore stain**

3- **Culture on blood agar**

4- **Catalase test**

5- **Motility test**

6- **Molecular methods**

Test	***C. perfringens***	***C. tetani***	***C. botulinum***
Gram stain	+	+	+
Spore stain	Does not sporulate on ordinary media	Round, terminal	Oval, subterminal
Colonial appearance on blood agar	Large, smooth, regular convex colonies, but may be rough and flat with an irregular edge. Usually has a double zone of β-haemolysis; produces lecithinase	Fine swarming growth (may be difficult to see) which may appear β-haemolytic	Large (3 mm), irregularly circular, smooth, greyssh, translucent with a fibrillar edge that may spread. Most strains are β-haemolytic; produces lipase
Catalase	-	-	-
Motility	-	+	+

Diagnostic Laboratory Tests:

1. Specimens:

Specimens consist of fresh sputum, gastric washings, urine, pleural fluid, cerebrospinal fluid, joint fluid, biopsy material, blood, or other suspected material,

2. Smears:

a. Ziehl - Neelsen staining
b. Auramine-Rhodamine fluorochrome stain

Principle

The fluorochrome dyes used in this stain complex to the mycolic acids in acid-fast cell walls. Detection of fluorescing cells is enhanced by the brightness against a dark background.

Method

a) Heat-fix slides at 80° C for at least 15 minutes or for 2 hours at 65° to 70° C.
b) Flood slides with auramine-rhodamine reagent and allow to stain for 15 to 20 minutes at room temperature.
c) Rinse with deionized water and tilt slide to drain.
d) De colourize with 0.5% acid-alcohol (70% ethanol and 0.5% hydrochloric acid) for 2 to 3 minutes.
e) Rinse with deionized water and tilt slide to drain.
f) Flood slides with 0.5% potassium permanganate for 2 to 4 minutes.
g) Rinse with deionized water and air dry.
h) Examine under low power (250×) for fluorescence.

Expected Results

Mycobacterium spp. fluoresce yellow to orange, depending on the filter system used.

3. Culture

Types of media used to cultivate *Mycobacterium*:

a) Agar based – growth within 10-12 days (e.g., Middlebrook media)
b) Egg based – growth within 18-24 days:
   Löwenstein - Jensen: contains salts, glycerol, and complex organic substances (e.g., fresh eggs or egg yolks potato flour) Malachite green is included to inhibit other bacteria.
   Specimens from nonsterile sites (decontaminated with NaOH, kills many other bacteria and fungi) neutralized with buffer, and concentrated by centrifugation.
   Löwenstein - Jensen inoculated and incubation is 35 - 37 °C in 5 - 10 % CO2 for up to 8 weeks,
c) Liquid media (e.g., Middlebrook and BACTEC).

4. Niacin test

Principle

The accumulation of niacin in an egg-based medium, the result of lack of an enzyme that converts niacin to another metabolite in the coenzyme pathway, is characteristic of M. tuberculosis and a few other species. Niacin is measured by a coloured end product.

Method

a) Add 1 ml of sterile distilled water to the surface of the egg-based medium on which the colonies to be tested are growing.
b) Lay the tube horizontally so that the fluid is in contact with the entire surface. Using a pipette, scratch or lightly poke through the

surface of the agar; this allows the niacin in the medium to dissolve in the water.

c) Allow the tube to sit for up to 30 minutes at room temperature. It can incubate longer to achieve a stronger reaction.

d) Remove 0.6 ml of the distilled water (which appears cloudy at this point) to a clean, 75 × 12 mm screw cap or snap top test tube. Insert a niacin test strip with the arrow down, following the manufacturer's instructions.

e) Cap the tube tightly and incubate at room temperature, occasionally shaking the tube to mix the fluid with the reagent on the bottom of the strip.

f) After 20 minutes, observe the colour of the liquid against a white background

Expected Results

Yellow liquid indicates a positive test result. The colour of the strip should not be considered when evaluating results. If the liquid is clear, the test result is negative. Discard the strip into an alkaline disinfectant (10% sodium hydroxide [NaOH]) to neutralize the cyanogen bromide.

5. **Nitrate reduction test**

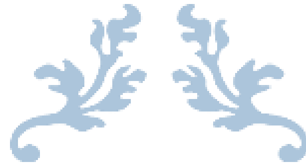
6. **Growth Inhibition by Thiophene-2-Carboxylic Acid Hydrazide (TCH).**

This test is used to distinguish *M. bovis* from *M. tuberculosis*, because only *M. bovis* is unable to grow in the presence of 10 mg/ml of TCH.

7. **DNA Detection**

The polymerase chain reaction holds great promise for the rapid and direct detection of *M. tuberculosis* in clinical specimens.

Species	Niacin	Growth on TCH	Nitrate reduction
M. tuberculosis	+	+	+
M. bovis	-	-	-



NEISSERIA

Lab ten

By

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DEPARTMENT OF BIOLOGY

COLLEGE OF SCIENCE

University of Baghdad

Lab 10: *Neisseria*

Lab diagnostic tests

1. Gram stain
2. Oxidase test.
3. **C**arbohydrate fermentation:

   Inoculation a loopful to Mueller Hinton agar + bromothymol blue pH 6 -7.6 containing 10% of lactose, sucrose, glucose, maltose, and fructose.
4. Streaking on chocolate agar for colonial morphology & pigmentation.
5. Nitrate reduction test.
6. Serology

Test	*N. gonorrhoeae*	*N. meningitides*
Gram stain	Gram negative diplococci	Gram negative diplococci
Glucose	+	+
Maltose	-	+
Fructose	-	-
Sucrose	-	-
Lactose	-	-
Pigments	Grayish white	Grayish white
CO2 requirement	Necessary	Necessary
Growth at 22°C	-	-
Growth at 35°C	+	+
NO3 reduction	-	+
Morphology	Smooth non capsulated	Transparent, flattened, mucoid if capsulated