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BIOCHEMICAL TESTS FOR THE IDENTIFICATION OF BACTERIA

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Introduction:

Bacteria are identified by different methods.Microscopic morphology is the primary character for any bacterial observation. It gives only shape, size, arrangement and staining characters. Several bacteria are similar morphologically unde microscope which may be with in genera, species or strains etc. Further identification is done by studying the cultural or growth characterson different culture media under different conditions. Further characterization of bacteria is done by metabolic or biochemical fermentation characters.

A given bacterial organism is studied for its ability to metabolize a given substrate as carbon or nitrogen otherwise any other nutrient material source. Ability of the organism under study for utilization of given substrate may be similar or different with other organisms. Similarly the organisms by its metabolic degradation of given substrate forms a product such product may be similar or different with other organisms. Depending on the organism's metabolic property to possess and operate a metabolic pathway makes it to degrade a given substrate and forms product or products. Such metabolic activities are tested under defined conditions of growth environ ment such as physical conditions(incubation conditions),chemical conditions(growth medium)etc. By examining such characters under similar conditions, the organisms are differentiated based on their substrate utilization and product formation. These biochemical test are used in identification unknown bacteria and these tests have much clinical importance in diagnostic laboratories

A biochemical test should be performed only on a pure culture of bacteria, isolated as a 'pure culture'. Biochemical tests for identification are not valid and will give misleading results unless the culture used in pure. A single colony should be sub-cultured into a liquid medium, which is usually peptone water unless the organism is fastidious (when a serum containing liquid medium may be used). After incubation for several hours to obtain a turbid growth, the liquid growth medium is sub-cultured into each of the biochemical test media appropriately selected for identification of the organism concerned. In addition, a check must be carried out on the purity of the inoculum by plating out the liquid growth on to a non-selective general purpose solid growth medium so as to obtain well separated colonies. This plate culture is known as purity plate.

The dictum

Use of a Pure culture of the bacterium for inoculation of test medium a must control for tests to be included.

Test to metabolism of carbohydrates & related compounds:

O/F test

Carbohydrate fermentation tests

Tests for specific break down products:

Methyl red test

V-P (acetoin production) test

Gluconate test.

Test to show ability to utilize a specific substrate:

Citrate utilization test.

Malonate utilization test

Gelatin liquefaction

Digestion of milk

Test for metabolism of protein and amino acids:

Indole test

Hydrogen sulphide production

A.A decarboxylase And arginine dihydrolase

Phenylalanine deamiase

Test for metabolism of lipid:

Hydrolysis of tributyrin

Test for enzymes:

Catalase test.

Oxidase test.

Urease test.

ONPG (β- galactosidase)test.

Nitrate reduction test.

Test for lecithinase and lipase.

Phenylalanine deaminase test.

Miscellaneous tests:

Potassium cyanide test

Litmus milk test.

1. Catalase test

Principle: To test for the presence of enzyme catalase.

Catalase Test: Catalase is a hemo protein found in most arobic & facultative anaerobic bacteria. Hydrogen peroxide forms as an oxidative end product of aerobic CHO metabolism which is lethal to cell. Catalase decomposes into H2O and O2

$2\ H_2O_2 \rightarrow 2\ H_2O + O_{2(nacent)}$

- **Procedures:**

Direct method- 3% H_2O_2 is added to the colonies on the plate. A consistent production of bubbles is a positive test.

Slide method- 30% H_2O_2 is used. The center of the colony to be tested is picked up with a wooden stick and placed on a slide and one drop of H_2O_2 is added. Production of bubbles indicate a positive test.

Tube method- 3% of H_2O_2 in a test tube the colonies are added immediate effecrvesence indicates a positive test.

Quality control - streptococcus spp. And Staph aureus.

Catalase test for Mycobacterium spp differentiation

- Some forms of catalases are inactivated at 68 C for 20 min.
- Heat stable catalase test: 30% H_2O_2 in a strong detergent solution (10% Tween 80).
- Semi-quantitative catalase test:

High catalase>45 mm of foam

Low catalase<45 mm of foam

Catalase is used for……..

Negative	Positive
Streptococcus Bacillus Listeria moncytogenes, corynebacterium Moraxella spp.	Staphyloccocus Clostridium Erysipelothrix

2. Oxidase test

Principle:

To determine the presence of the oxidase enzymes. The test really describes the presence of cytochrome c.

The cytochromes are iron containing hemoproteins that act as the last link in the chain of aerobic respiration by transferring electrons (hydrogen) to oxygen, with the formation of water.

2 reduced cytochrome c + 2H + ½ O_2 2 oxidised cytochromes + H_2O

The test is helpful in screening colonies suspected of being one of the Enterobacteriaceae (all negative) and identifying genera such as Aeromonas, Pseudomonas, Neisseria, Capylobacter and Pasturella (positive).

A positive oxidase result consists of a series of reactions in which an autooxidizable component of the cytochrome system is the final catalyst.

The cytochrome oxidase test uses certain reagent dyes, such as p-phenylenediamine dihydrochloride, that substitute for oxygen as artificial electron acceptor. In the reduced state the dye is colorless but in the presence of cytochrome oxidase oxygen p-phenyldiamine is oxidized forming indophenol blue.

n,n-dimethyl-p-phenylene diamine + naphthol + $O_2 \xrightarrow{enzyme}$ indophenol blue + water.

Media and reagents:

- Tetramethyl-p-phenylenediamine dihydrochloride, 1% (Kovac's reagent)
- Dimethy-p-phenylenediamine dihydrochloride, 1%(Gordon and McLeod's reagent).
- Reagent A, 1% a naphthol in 95% ethylalcohol and reagent B 1% p-aminodimethylaniline HCL(oxalate)
- Carpenter, suhrland and Morrison reagent 1% p-aminodimethyl aniline oxalate.
- Oxidase impregnated discs.
- Kovac's reagent is less toxic and extremely sensitive compared to dimethyl compound but more expensive. Gordon's reagent is more stable than Kovac's.
- P-aminodimethylaniline oxalate is extremely stable.

Procedure:

- Direct plate technique: 2 or 3 drops of reagent directly added to the isolated colonies on plate medium. Positive reaction pink to purple almost black within 10 sec. Result within 10-60 sec delayed result.
- Cytochrome oxidase does not react direly with the reagent but oxidizes cytochrome c which in turn oxidizes the reagent.

2reduced cytochrome c + 2H + ½ O_2 2 oxidised cytochromes + H_2O

2 oxidised cytochromes + Reagent colored compound

- *2) Indirect paper strip procedure* – Few drops of the reagent are added to a filter paper and a loop full of suspected colony is smeared into the reagent zone of the filter paper.
- Quality Control: E.coli-negative control
- Pseudomonas aeruginosa – positive control.
- Precautions:
- All reagents should be freshly prepared just prior to use, once in solution they become deactivated rapidly.
- Do not perform oxidase test on colonies growing on medium containing glucose as its fermentation will inhibit oxidase enzyme activity: Oxidase test of GNB should be done on nonselective media.
- Use of platinum loop for removing colonies advocated as presence of traces of iron also catalyzes oxidation.

3. Indole test

- Principle: To determine the ability of an organism to split indole from tryptophan molecule.
- Tryptophan an amino acid is converted by an enzyme *tryptophanase* into, indole, pyruvic acid, ammonia and energy.

- Pyruvic acid is metabolized either by glycolytic pathway or can enter Kreb's cycle to release CO_2, H_2O and energy. NH_3 is used to make new amino acid.

Chemistry of the reaction: Indole present combines with the aldehyde in the reagent to give a red color in the alcohol layer. The color is based on the presence of pyrrole structure present in the indole. (Quinoidal red-violet compound)

- The alcoholic layer extracts and concentrates the red color complex.

P-Dimethylaminobenzaldehyde + indole → red-violet color

Indole test is used for.....

Positive	Negative
Edwardsiella Eshcerichia coli H. Influenzae Proteus sps	Salmonella Klebsiella-Enterobacter Haemophilus spp Proteus mirabilis

4. Methyl red test

Principle:

To test the ability of an organism to produce and maintain stable acid end products from glucose fermentation, and to overcome the buffering capacity of the system.

M.R. test is a *quantitative test for acid production requiring positive organisms to produce strong acids, form glucose fermentation.*

- Methyl red is a ph indicator with a range between 6.0 (yellow) and 4.4 (red). The ph at which methyl red detects acid is considerably lower than the pH of other indicators.

E.M.glycolytic

α – D-glucose → pyruvic acid

pathyway

pyruvic acid mixed acids CO_2

Methyl red methyred

Yellow pH 6.0 pH 4.4

Medium employed

Clark and Lubs medium (MR/VP Broth), pH 6.9

Bufered peptone 0.5%, glucose, dipotassium phosphate buffer.

Incubation – 35°C for 48 hr or 30°C for 3- days.

Aseptically by pipette remove 2.5 ml of inoculated medium and add 5 drops of methyl red indicator

- MR positive: culture sufficiently acid to allow the methyl red reagent to remain distinct red color (pH 4.4). At the surface of the medium.
- MR negative: Yellow color
- Delayed reaction: orange color, Continue incubation to 4 days and repeat the test.

Precautions

- No attempt should be made to interpret a methyl red result before 48 hrs of incubation. As it may be falsely positive purpose of the test is to differentiate E. coli(+) from Klebsella (-) and Enterobacter(-)

Yersinia spp(+) from other gram negative non-enteric bacilli(-).

To aid in the identification of Listeria monocytogenes(+).

5. Voges-Proskauer test

Named after two microbiologists.

Principle:

To determine the ability of some organisms to produce a neutral end product, acetyl methyl carbinol(acetoin) from glucose fermentation.

Pyruvic acid a pivotal compound in glucose metabolism, is further metabolized thru various metabolic pathways. One such pathway results in the production acetoin.

Acetoin may be converted into butanediol by reduction or by oxidation into diacetyl in the presence of oxygen and 40% KoH.

α – D-glucose $\xrightarrow{F.M.\,pathway}$ pyruvic acid

Pyruvic acid acetoin $\xrightarrow[KOH]{butylene\,glycol\,pathway}$ acetoin + carbondioxide diacetyl

Diacetyl anaphhol + guanidine group→condensation pink product

Aseptically remove and aliquot for VP determination.

Barritt's test: 2.5ml

O'Meara test: 1.0 ml

Add first reagent A-0.6 ml or barritt's reagent

Second reagent B- 0.2 ml

OR

1 ml of O'Meara's reagent

Shake tubes gently 30 sec to 1 min observe after 10-15 min for the production of pinkish red color at the surface of medium

Precautions

- The order of adding Barritt's VP reagents is important. First α naphthol to be added followed by 40% KOH other wise a false negative reaction occurs.
- An exact amount of 0.2 ml of 40% KOH should not be exceeded as it may mask a weak VP positive reaction by exhibiting a copper like color due to the reaction with α naphthol alone.

6. Citrate test

Principle:

To determine if an organism is capable of utilizing citrate as the sole carbon and energy source for growth and an ammonium salt as the sole source of nitrogen.

The medium used for citrate fermentation also contains inorganic ammonium salts. An organism that is capable of utilizing citrate also utilizes the ammonium salts as its sole nitrogen source breaking ammonium salts to ammonia with resulting alkalinity.

Medium used:

Koser's liquid medium:

Sodium citrate, sodium chloride, ammonium and potassium dihydrogen phosphate.

Simmon's citrate medium:

This is a modification of Koser's medium with agar and an indicator bromothymol blue added.

Method:

Inoculate from a saline suspension. Incubate for 24-48 hrs.

Koser's medium – positive - turbidity

Negative – no turbidity

Simmon's method – positive – blue color and streak of growth.

Negative – original green color & no growth.

Purpose of citrate test

Salmonella(+) Edwardsiella (-)

Klebsiella (+) Escherichia coli(-)

Bordetella spp (+) Bordetella pertussis(-)

7. Urease test

Principle:

To determine the ability of an organism to split urea, forming two molecules of ammonia by the action of the enzyme urease with resulting alkalinity.

Urea is a diamide of carbonic acid. Urease is an enzyme possessed by many spp of bacteria that can hydrolyze urea to form ammonia & CO_2 & HO_2. the ammonia reacts in solution to form ammonium carbonate resulting in alkalinization and an increase in the pH of the medium.

Urea + HO_2 urease →ammonia + carbondioxide

Phenolphthalein ammonia →phenophthalein

Media employed:

1. Rustigian & Stuart's urea broth → yeast extract, mono potassium phosphate, disodium phosphate, urea, phenol red.
2. Christensen's urea agar – peptone, sodium chloride, mono potassium phosphate, glucose, urea, phenol red and agar.

Procedure:

Inoculate the broth/ agar and incubate at 35C and observe at 8, 12, 24 and 48 hrs.

Positive – intense pink color through out the slant.

Negative – no color change. (buff to pale yellow)

Degree of hydrolysis

1.4+; entire tube pink-red

2.2+; slant pink, butt no change.

3. weakly +; top of slant pink, remainder no change.

Purpose : Klebsiella(+), form Escherichia(-)

Proteus (+) form Providentia(-)

Crytococcus (+), Helicobacter pylori(+) very rapid.

8. Coagulase Test

Principle:

- ❖ To test the ability of an organism to clot plasma by the action of the enzyme coagulase (Staphcoagulase).
- ❖ A positive coagulase test is usually the final diagnostic criterion for the identification of Staphylococcus aureus.
- ❖ Coagulase is a protein having a prothrombin like activity capable of converting fibrinogen into fibrin, which results in the formation of a visible clot.

Coagulase is present in two forms, bound and free each having different properties that require the use of separate testing procedure.

- **Bound coagulase (slide test):**

  Bound coagulase also known as clumping factor is attached to the bacterial cell wall. Fibrin stands are formed between the bacterial cells when suspended in plasma causing them clump into visible aggregates

- ➢ **Free coagulase: (Tube test):**

Free coagulase is a thrombin like substance present in culture filtrates. When a suspension of coagulase producing organisms is prepared in plasma in a test tube, a visible clot forms as a result of coagulase reacting with a serum substance (coagulase- reacting factor) to form a complex which in turn reacts with fibrinogen to produce the fibrin clot.

- ➢ Media and reagents:
- ➢ Rabbit plasma with EDTA.

Procedure:

Slide test:

- Place two drops of saline in two circles drawn on a glass slide. Gently emulsify test organism in liquid in each circles. Place a drop of plasma in the suspension in one circles and drop of water to the other circle.

Mix with a wooden applicator stick. Observe for agglutination. The saline control should be smooth and milky.

- Positive test: marked clumping within 5 to 20 sec.
- Delayed positive test: clumping after 20 sec and up to 1 min.
- All strains producing negative slide tests must be tested with tube coagulase test.

Tube test:

- Emulsify a small amount of the test organism in a tube containing 0.5 ml of plasma. Incubate the tube at 35C for 4 hrs and observe for clot formation by gently tilting the tube. If no clot is observed, reincubate the tube at room temp and read again after 18 hrs.
- Positive test: Clot or distinct fibrin threads
- 1. Complete: clot through out the tube.
- 2. Partial clot does not extend throughout fluid column. Any degree of clotting is considered positive.

9. Nitrate reduction Test

Principle:

To determine the ability of an organism to reduce nitrate to nitrites or free nitrogen gas.

All enterobacteriaceae except some biotypes of Pantoea, serratia and Yersinia demonstrate nitrate reduction. Also helps in identifying members of Haemophilus, Neisseria and moraxella.

Organisms demonstrating nitrate reduction have the capability of extracting oxygen from nitrates to form nitrates and other reduction products.

$NO_3 + 2e^- \rightarrow NO_2 + H_2O$

- Media employed:

1. Nitrate broth pH 7.0 {potassium nitrate, peptone and beef extract}
2. Nitrate agar.

Regents employed:

Reagent A: α – naphthylamine (To prepare dissolve the chemical in 5N acetic acid)

Reagent B: Sulfanilic acid (p-aminobenzene sulfonic acid)

- Inoculate the medium and incubate for 35C for 24 hrs sometimes up to 5 days.
- After incubation, alpha-napthylamine and sulfanilic acid are added. These two compounds react with nitrite and turn red in color, indicating a positive nitrate reduction test. When nitrates are reduced to nitrites, nitrites reacts with the two reagents and forms a diazonium compound p- sulfobenzene-azo- α – naphthylamine.
- If there is no color change at this step, nitrate is absent. If the nitrate is unreduced and still in its original form, this would be a negative nitrate reduction result. However, it is possible that the nitrate was reduced to nitrite but has been further reduced to ammonia or nitrogen gas. This would be recorded as a positive nitrate reduction result.
- To distinguish between these two reactions, zinc dust must be added. Zinc reduces nitrate to nitrite. If the test organism did not reduce the nitrate to nitrite, the zinc will change the nitrate to nitrite. The tube will turn red because alpha-naphthylamine and sulfanilic acid are already present in the tube. Thus a red coor after the zinc is added indicates the zinc found the nitrate unchanged. The bacteria was unable to reduce nitrate. This is recorded as a negative nitrate reduction test.
- If however, the tube does not change color upon the addition of zinc, then the zinc did not find any nitrate in the tube. That means the test organism converted the nitrate to nitrite and then converted the nitrite to ammonia and/or nitrogen gas. Thus no color change upon the addition of zinc is recorded as a positive nitrate reduction test.

10. Kligler's iron agar/triple sugar iron agar tests

Principle:

- To determine the ability of an organism to attack a specific carbohydrate incorporated in the medium with or without the production of gas, along with the determination of possible hydrogen sulfide production.
- KIA and TSI are tubed differential media. KIA contains two carbohydrates ; lactose, 1.0% concentration and glucose in a .01% concentration. TSI has a third carbohydrate sucrose in 1.0% concentration.
- Carbohydrate fermentation can occur with or without gas production.
- Fermentation occurs both aerobically on the slant and anaerobically in the butt.
- TSI reaction are primarily for the identification of members of the enterobacteriaceae.
- There are three basic fermentation patterns observed
- 1. Glucose fermentation only 2. fermentation of both glucose and lactose 3. failure to ferment both.
- TSI tubes to be interpreted at the end of 18-24 hrs of incubation. Earlier or delayed interpretations are invalid.

Interpretations:

- Alkaline/acid fermentation of glucose only. Red/yellow color.
- Acid/acid fermentation of glucose and lactose, yellow/yellow.
- Alkaline/alkaline neither glucose nor lactose fermented red/no change in color
- Alkaline/no change neither glucose nor lactose fermented, peptones utilized. Growth only on change in color/growth only no color change.
- Gas production is evident as bubbles or splitting of the medium.
- An H_2S organism may produce so much of the black precipitate (ferrous sulphide) that the acidity produced in the butt is completely masked. However, if H_2S is produced, an acid condition does exist in the butt even if it is not observable.

Purpose; fermentation patterns are specific for genera and spp of enterobacteriacea

Acid/acid with or without gas

Escherichia

Klebsiella

Citrobacter

Enerobacter

Yersinia enterocolotica

Hafnia

Acid/acid H_2S Citrobacter freundii

Alkaline/acid with or without gas

Salmonella

Proteus

shigella

yersinia

alkaline/alkaline or alkaline/no change

alkaligenes faecalis

11. Carbohydrate fermentation tests

Principle: To determine the ability of an organism to ferment (degrade) a specific carbohydrate incorporated in a basal medium producing acid or acid with visible gas.

- Purpose: Fermentation patterns are specific for each group or spp.
- All enterobacteriacea are glucose fermenters.
- E.coli, Klebsiella and Enterobacter are glucose and lactose fermenters.
- Listeria are salicin positive and Listeria are salicin negative.
- Staphylococcus aureus – mannitol.

- Neiseria lactamica – lactose
- E.coli) 157 H7 – sorbitol.

Carbohydrates include not only sugars but poly hydric alcohols like mannitol and dulcitol.

The fermentation end products are: two gases hydrogen and carbondioxide, few acids lactic, acetic and formic acids etc, a few alcohols isopropyl alcohol, ethyl alcohol and one ketone β hydroxy butyric acid.

- Media employed:
- Broth base with peptone, beef extract, Nacl with 1% sugar and an indicator with phenol red, Andrade's indictor bromocresol purple.
- A variety of carbohydrates may be utilized. Generally utilize 8 to 10 sugars. Most often employed are 1) glucose 2) lactose 3) sucrose 4) mannitol 5)dulcitol 6)salicin 7)adonitol 8)inositol 9) sorbitol 10)arabinose 11)raffinose 12)rhamnose 13)xylose 14)inulin etc.
- At times serum peptone fermentation media or serum peptone fermentation agar media may be used
- A durham's tube to be placed inverted in the tube of glucose.
- The test organism should be inoculated in the battery of sugars a loopful or one drop and incubated at 37C for 24 hrs.
- Look for acid and gas.

Ph indicator	acid(fermentation)	alkaline(negative)
Phenol red	Yellow	Pinkish – red
Andrade's	Pinkish red	Yellow

12. Oxidation – fermentation test

To determine the oxidative or fermentative metabolism of a carbohydrates or its non-utilization purpose:

- Enterobacteriacae glucose fermenters
- Pseudomonas spp glucose oxidizers
- Alkaigenes faecalis inert neither fermentor nor oxidizer

- Micrococcus spp usually oxidizers
- Staphylococcus spp fermenters.

Interpretation:

Oxidation: Open tube Yellow(acid) and scaled tube (green)

Fermentation (an aerogenic) open tube Yellow and scaled tube yellow

Neither fermentation or oxidation both tubes blue or green

API test system

The Analytical profile Index(API) is a miniaturized panel of biochemical tests compiled for identification of groups of closely related bacteria. Different test panels are prepared in dehydrated forms which are reconstituted upon use by addition of bacterial suspensions. After incubation, positive test results are scored as a seven-digit number(profile). Identity of the bacterium is then easily derived from the database with the relevant cumulative profile code book or software

- API 20E presented herein is a biochemical panel for identification and differentiation of members of the family Enerobacteriaceae. Other API panels for other groups of bacteria, such as staphylococci and streptococci, are also available in the same format.
- In API 20E for identification of members of the family Enterobacteriaceae, the plastic strip holds twenty mini-test chambers containing dehydrated media having chemically – defined compositions for each test
- These include:
- ONPG: test for b-galactosidase enzyme by hydrolysis of the substrate o- nitrophenyl-b-D-galactopyranoside
- ADH: decarboxylation of the amino acid arginine by arginine dihydrolase
- LDC: decarboxylations of the amino acid by lysine by lysine decarboxylase
- ODC: decarboxylations of the amino acid ornithine by ornithine decarboxylase
- CIT: utilization of citrate as sole carbon source
- H_2S: production of hydrogen sulfide

- URE: test for the enzyme urease
- TDA: detection of the enzyme tryptophan deaminase
- IND: production of indole from tryptophan by the enzyme tryptophanase. Indole is detected by addition of Kovac's reagent
- VP: the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylenes glycol pathway GEL: test for the production of the enzyme gelatinase which liquefies gelatin.
- GLU: fermentation of glucose (hexose sugar)
- MAN: fermentation of mannose (hexose sugar)
- INO: fermentation of inositol (cyclic polyalcohol)
- SOR: fermentation of sorbitol (alcohol sugar)
- RHA: fermentation of rhamnose (methyl pentose sugar)
- SAC: fermentation of sucrose (disaccharide)
- MEL: fermentation of melibiose (disaccharide)
- AMY: fermentation of amygdalin (glycoside)
- ARA: fermentation of arabinose (pentose sugar)
- The OX test is at test for cytochrome oxidase which is preformed separately from the above tests. It is done using a portion of a bacterial colony on a paper strip impregnated by the oxidase regagent N,N,N',N'-tertramethyl phenylenediamine which turns blue if cells possess oxidase enzyme.

- All test chambers are rehydrated by inoculation with a saline suspension of a pure culture of the bacterial strain subjected to identification (or a manufacturer – supplied suspending medium). After incubation in a humidity chamber for 18 to 24 hours at 37^0C, the color reaction are read. The results of the test reactions (plus the oxidase reaction which is done separately) are converted to a seven-digit code. The code can be then be

looked up in the database book or software or fed into the manufacture's database via touch-tone telephone where the computer voice gives the genus and species identification of the test microorganism.

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